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Dissertação de Mestrado

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Dissertação orientada pelo Prof. Doutor Arsénio Fialho, orientador externo, Instituto Superior Técnico (IST) e pela Prof. Doutora Maria Teresa Rebelo, orientador interno, Faculdade de Ciências da Universidade de Lisboa (FCUL)

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List of abbreviations

aGM1 - asialo-GM1

ASL - airway surface liquid

Bcc - *Burkholderia cepacia* complex

CDS - coding sequence

CF - cystic fibrosis

CFF - Cystic Fibrosis Foundation

CK - cytokeratin-13

CFRT - cystic fibrosis transmembrane conductance regulator

DNA - deoxyribonucleic acid

ECM - extracelular matrix

EnaC - epithelial sodium channels

EDTA - ethylenediamine tetraacetic acid

EGTA - ethylene glycol tetraacetic acid

EPS - exopolysaccharide

ER – endoplasmatic reticulum

ET12 - Edinburgh-Toronto lineage

GM1 - ganglioside 1

GM2 - ganglioside 2

GPI - glycosylphosphatidylinositol

HBS - hepes buffered saline

HGF-R - hepatocyte growth factor receptor

IgA - immunoglobulin A

IgD - immunoglobulin D

IgG - immunoglobulin G

IL-8 - interleukin 8

LB - Luria Bertani broth

LPS - lipopolysaccharide

MβCD - methyl -β-ciclodextrin

MEM - minimum essential medium

MIC - minimal inhibitory concentration

MLSA - multilocus sequence analysis

MLST - multilocus sequence typing

MOI - multiplicity of infection

PCR - polymerase chain reaction

PBS - phosphate buffered saline

QS - quorum sensing

ROS – reactive oxygen species

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

rRNA - ribossmal ribonucleic acid

RNA - ribonucleic acid

ST - sequence type

TAA - trimeric autotransporter adhesin

TCE - trichloroethylene

Tp - trimethoprim

TTSS - type III secretion systems

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Resumo

O complexo *Burkholderia cepacia* (Bcc) é constituído por, pelo menos, 18 espécies do género *Burkholderia*, estas bactérias são agentes patogénicos oportunistas capazes de causar infeções severas e, frequentemente, morte em doentes com fibrose quística (CF). A fibrose quistica é provocada pela mutação do gene codificante do regulador de condutância transmembranar (CFRT) que causa a sua ausência na membrana apical das células epiteliais. Esta doença é caracterizada pela inflamação e infeção crónica das vias respiratórias por *Pseudomonas aeruginosa e Burkholderia cepacia.*

Agentes patogénicos como *B. cepacia* apresentam muitos fatores de virulência que os ajudam a estabelecer infeção e a invadir células hospedeiras. Entre os muitos fatores já estudados estão as adesinas triméricas autotransportadas (TAAs). Estas proteínas são responsáveis pela adesão às células hospedeiras e, também pela formação de biofilme, invasão e sobrevivência destas bactérias dentro de células hospedeiras. Estas adesinas são o único mecanismo de aderência a células hospedeiras conhecido em *Burkholderia*, para além da adesina de 22KDa associada a pili, também já estudada. No entanto, apesar de já demonstrada a importância das TAAs para capacidade de adesão e invasão de *B. cenocepacia* a porta de entrada utilizada por estes patogéneos ainda não é conhecida, e, por isso, neste trabalho as jangadas lípidicas foram estudadas como uma possível opção.

A análise do genoma da estirpe *B. cenocepacia* J2315 revelou a existência de um agrupamento de genes no qual a lipoproteína de membrana BCAM0220 e da adesina BCAM0219 formam um operão, sugerindo, portanto, a assistência da lipoproteina na biogénese da adesina BCAM0218, como o já estudado entre as proteínas SadA e SadB em *Salmonella*.

Neste trabalho foi possível perceber que as jangadas lípidicas não são utilizadas como portas de entrada na ligação das TAAs na infeção de células epiteliais por *B. cenocepacia*. No entanto, não foi possível estudar a sugerida relação entre as proteínas BCAM0220 e BCAM0219, pois as ferramentas necessárias não foram obtidas.

Palavras-chave: *Burkholderia cenocepacia*; adesina trimérica autotransportada; fibrose quística; jangadas lípidicas

Abstract

The *Burkholderia cepacia* complex (Bcc) comprises, at least, 18 species of the *Burkholderia* genus, these bacteria are opportunistic pathogens able to cause severe and often lethal respiratory infections in cystic fibrosis (CF) patients. CF is a genetic disorder that causes the lack of the transmembrane conductance regulator (CFRT) protein at the apical membrane of epithelial cells. This disease is characterized by chronic airway infection, which can be caused by a variety of microorganisms, such as *Pseudomonas aeruginosa* and *Burkholderia cepacia,* and persistent airway inflammation.

Pathogenic bacteria, as *Burkholderia* bacteria possess a vast array of virulence determinants that are used to cause infection. Although, pathogenicity in these bacteria is still poorly understood many virulence determinants have already been described, among them there are the trimeric autotransporter adhesins (TAAs). The TAAs are responsible for not only adherence, but biofilm formation, invasion and survival within eukaryotic cells, as well. Additionally, the only other virulence factor responsible for adhesion already described in *Burkholderia* is the 22KDa cable pili associated adhesin. Previous studies demonstrated that TAAs are necessary for *B. cenocepacia*'s ability to adhere and invade airway epithelial cells, nevertheless, the gateways that these bacteria use to do so have yet not been described, therefore, in this study, lipid rafts were exploited as a possibility.

Furthermore, the study of the genome of the *B. cenocepacia* J2315 strain revealed the existence of a putative adhesin cluster in which the TAA *BCAM0219* gene and the outer-membrane lipoprotein BCAM0220 are an operonic structure suggesting a possible assistance of the *BCAM0220* lipoprotein in BCAM0219 TAA biogenesis, as it has been described for the SadA and SadB *Salmonella* proteins.

In this study, it was possible to determine that lipid rafts are not used as gateways, but, unfortunately, it was not possible to study BCAM0220 and BCAM0219 interaction as the necessary tools were not obtained.

Key-words: *Burkholderia cenocepacia*; trimeric autotransporter adhesin; cystic fibrosis; lipid rafts

1. Introduction

1.1 *Burkholderia cepacia* complex

The *Burkholderia* genus contains a group of versatile gram-negative bacteria that are often found in soil, water and plant rhizopheres. Some of its members are able to able to degrade major xenobiotic pollutants, enhance crop growth and survive in man-made products as pharmaceuticals.

Within this genus there's the *Burkholderia cepacia* complex (Bcc) a group of closely related species of human opportunistic pathogens, capable of causing chronic and severe infection especially in cystic fibrosis and immunocompromised patients, but, in contrast, it cannot affect healthy individuals (Coenye & Lipuma, 2003; Mahenthiralingam et al., 2008; O'Sullivan & Mahenthiralingam, 2005).

1.2 Taxonomy

In 1950, Burkholder described *Pseudomonas cepacia* as the responsible agent of bacterial onion rot (Burkholder, 1950). However, with the advent of new techniques as rRNA-DNA hybridization and various rRNA gene sequencing methods taxonomists realized that the *Pseudomonas* genus comprised five species clusters. Then, as consequence *Pseudomonas cepacia* and other species were transferred to the new *Burkholderia* genus. Later, several researchers noted a remarkable diversity among strains identified as *Burkholderia cepacia* using traditional and molecular identification approaches (Coenye et al., 2001). In fact, this single species was shown to consist of at least five different genetic species designated as genomovars, in consequence, the group of the five species was named the *B. cepacia* complex (Bcc)(Vandamme et al., 1997).

Numerous methods were used to establish correct relationships between strains and improve their identification. Among the genotyping methods applied there're the 16S rRNA and *recA* gene based analyses as well as the MLSA (multilocus sequence analysis) and MLST (multilocus sequence typing) approaches. The 16S rRNA and *recA* gene based analysis both confirmed phylogenetic relatedness of Bcc complex members, nevertheless, the 2% intraspecies diversity in their 16S rRNA gene overlapped *recA* gene restriction profiles, rendering these methods ineffective (Coenye et al., 2001; Vandamme & Dawyndt, 2011).

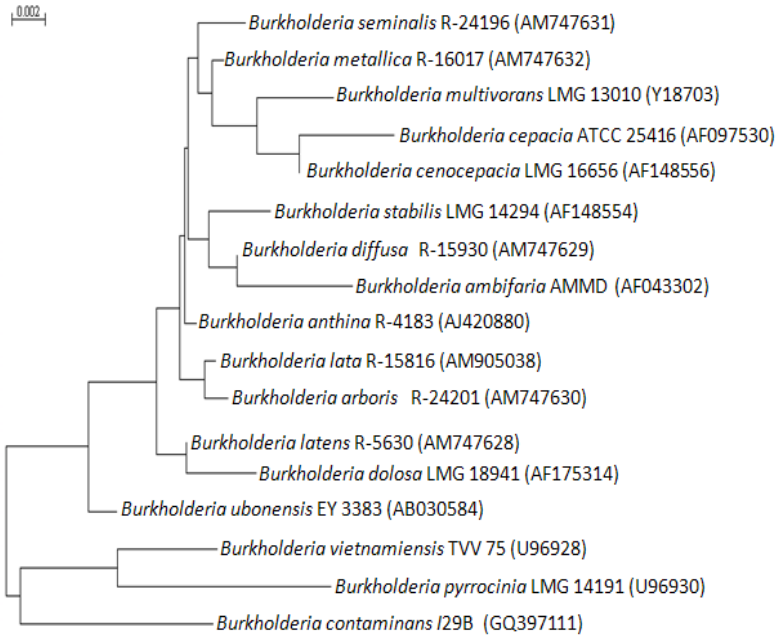

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Fig. 1: Bcc complex 16S rRNA based phylogenetic tree.

Instead, the MLSA approach uses allelic profiles nucleotide sequences capable to reveal similarities between strains of different species and genera. In contrast, the MLST approach can be specifically developed to measure similarities and differences, that make it able to clearly differentiate all Bcc closely related species and differentiate strains (Baldwin et al., 2005; Vandamme & Dawyndt, 2011). These allowed establishing the Bcc complex in seventeen species **(Fig 1).**

Furthermore, not only by *recA* gene sequence analysis the *B. cenocepacia* was subdivided into four phylogenic clusters IIIA to IIID, but almost all clinically relevant isolates belong in the IIIA and IIIB groups but, also, MLST analysis of the Bcc has led to understand that the Edinburgh-Toronto lineage (ET-12), a notorious epidemic strain, to which belongs the species *Burkholderia cenocepacia* K-56, appears to fit within five different sequences types (ST), being the ST-28 and ST-32 strains the accounted for multiple globally distinct locations (Mahenthiralingam et al., 2008).

Finally, *B. pseudomultivorans* a new species within the Bcc complex was just identified *by* using some of the analysis methods referred as *recA,* 16S rRNA sequence analysis and MLSA (Peteers et al., 2013).

1.3 Ecology and environmental diversity

The *Burkholderia* species complex can be found in soil, water and the rhizosphere of plants,

where they develop different types of non-pathogenic interactions with plants by colonizing its roots, leaves and stems (Coenye & Vandamme, 2003). These species are found in diverse habitats because of its assumed capacity to use a wide range of compounds as carbon sources. In fact, this species metabolic adaptability is enabled by its multireplicon genome that harbours a vast array of insertion sequences that promote genomic plasticity (Chiarini et al., 2006).

However, Bcc species are disproportionately distributed in their various habitats; for instance *B. cenocepacia* is prevalent in cystic fibrosis (CF) lung whereas *B. ambifaria* is most common in plant rhizosphere, like in both maize and tomato rhizosphere as well as associated with sugarcane (Caballero-Mellado et al, 2007; Castro-González et al., 2011; Chiarini et al., 2006; Ramette et al., 2005) **(Tab. 1)**. Most of the rhizosphere associated species have the ability of nitrogen fixation, to nodulate legumes and to degrade aromatic compounds, as well as the potential for bio inoculation and bio control (Suárez-Moreno et al., 2012).

Tab. 1: *B. cepacia* complex species diverse sources (Chiarini et al., 2006;Vial et al., 2011).

Species	Source	CF	Non -CF
B. ambifaria	Rhizosphere,soil,humans	X	X
B. anthina	Rhizosphere, soil ,water, hospital, environment, humans	X	X
B. cenocepacia	Rhizosphere, soil,water, hospital, environment, humans	X	X
B. cepacia	Rotten onion,rhizosphere, soil,water, humans	X	X
B. dolosa	Rhizosphere,humans	X	
B. multivorans	Rhizosphere, soil,water, humans	X	X
B. pyrrocinia	Rhizosphere, soil,water, humans	X	
B. stabilis	Hospital environment, humans	X	X
B. vietnamiensis	Rhizosphere, soil,water, humans	X	X
B. ubonensis	Soil		
B. latens	No environmental strain reported	X	
B. diffusa	Soil, water	X	X
B. arboris	Rhizosphere, soil, water	X	X
B. seminalis	Rice rhizosphere	X	
B. metallica	No environmental strain reported	X	
B. contaminans	Soil, water, animal	X	X
B. lata	Soil, water, flower	X	X

All species from the Bcc complex have been isolated from CF sputum cultures, with the exception of *B. ubonensis,* nevertheless, some species are more often found in CF patients than others. *B. cenocepacia* and *B. multivorans,* despite showing an highly disproportionate distribution within clinical isolates from CF and non-CF patients, are the most frequently found species, together accounting for at least 70% of infected patients in the United States, however, *B. cenocepacia* is the most commonly recovered species from CF patients across the world **(Fig.2)** (Lipuma et al., 2010; Reik et al., 2005).

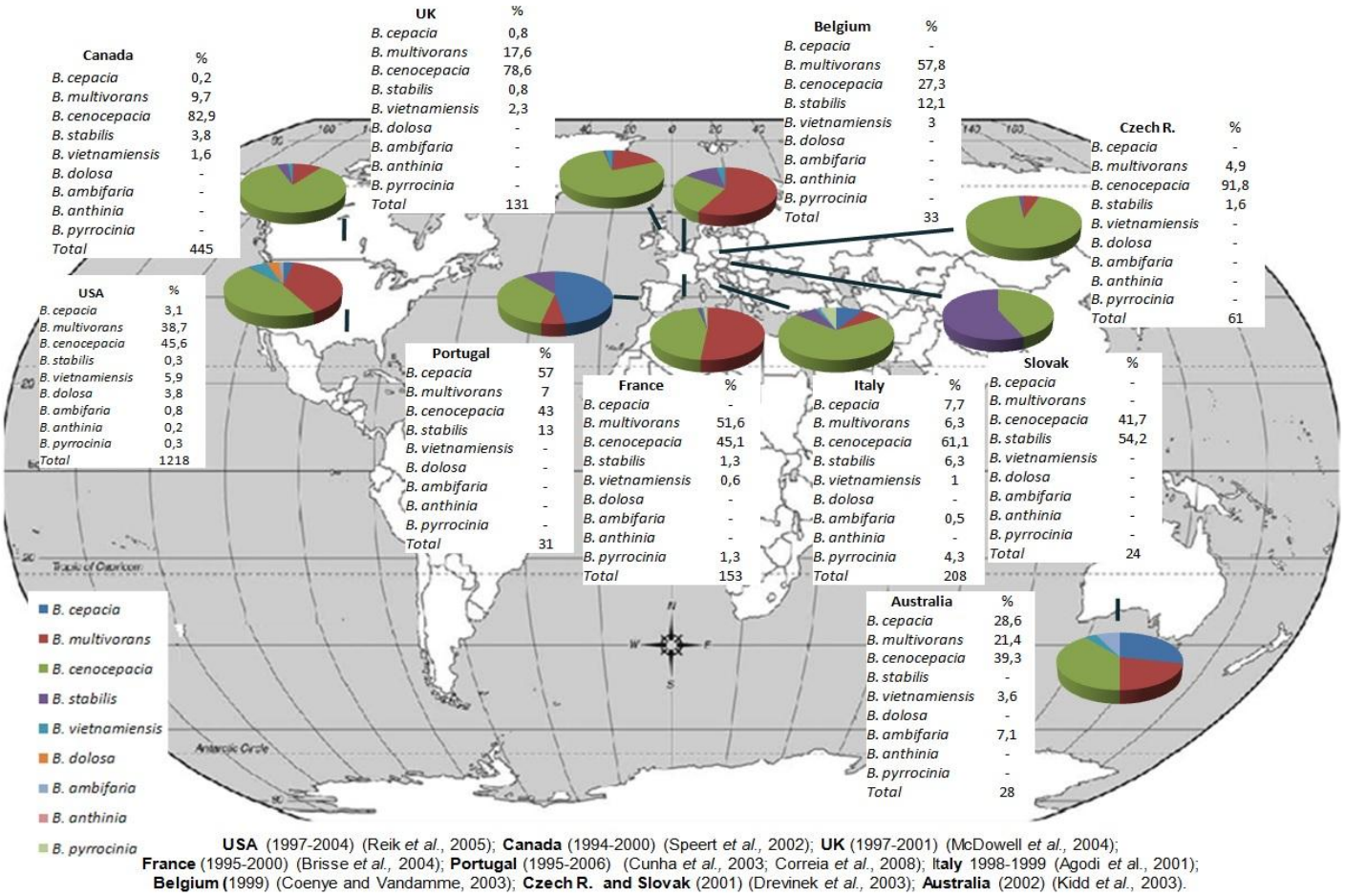


Fig. 2: Worldwide distribution of Bcc species in CF patients selected populations (Mil-Homens, 2012).

However, it is also important to note that *B. gladioli*, phenotypically close but not phylogenetically a member of the Bcc complex, is now more regularly found in CF patients across USA than most of the Bcc complex species, and *B. cepacia* is comparatively seldom found (Lipuma, 2010). Also, there are some *Burkholderia* bacteria that even by genetic analyses cannot be properly assigned to one of the current species; therefore, these strains likely belong to new species within the Bcc complex. Nevertheless, although Bcc complex species are frequently found in CF the most commonly found bacterial species is *Pseudomonas aeruginosa*, and, for example, in the United States, in 2008, 52.5% of patients included in the Cystic Fibrosis Foundation (CFF)

Patient Registry were reported to have had *P. aeruginosa* recovered from sputum culture (Lipuma, 2010).

Furthermore, *B. cenocepacia* and *B. multivorans* infection incidence has changed during the last years as, while *B. cenocepacia* previously accounted for the majority of *Burkholderia* infection in CF, now both in the United States and the United Kingdom the recovery of *B. multivorans* surpasses that of *B. cenocepacia* (Reik et al., 2005; Govan & Jones, 2007). The reasons for the predominance of these two species among CF patients are unknown in spite of the very close phylogenetic relatedness of species within the Bcc complex (Reik et al., 2005). However, the disproportion between *B. cenocepacia* and *B. multivorans* and the other species representation suggests a better capability of human infection (Lipuma et al., 2001; Reik et al., 2005).

Lastly, several studies identified common strains of *Burkholderia* bacteria in CF patients among them are epidemic ET12 strains, which are prevalent in the United Kingdom and eastern Canada, and other specific strains as the ST04 and the CZ1 were recovered from CF patients in Canada and Prague, respectively (Lipuma, 2010).

1.4 Cystic fibrosis physiology

Cystic fibrosis is the most common lethal autosomal recessive genetic disorder in Caucasians population caused by the cystic fibrosis transmembrane conductance regulator (CFRT) gene mutation, that results in the absence of the CFRT protein at its proper cellular location, the apical membrane (Boucher, 2004; Lyczak et al., 2002) . The majority of CF mutations are associated with the CFRT's protein misfolding, the most common mutation, the ΔF508 mutation was the first to show problems in polypeptide maturation and translocation to the suitable cellular site (Boucher, 2004).

This disease is characterized by sinopulmonary chronic infection, which can be caused by a variety of microorganisms, such *P. aeruginosa, Staphylococcus aureus* and members of the Bcc. Such microorganisms are often, capable of causing life-threatening respiratory tract infections and, ultimately, 80 to 90% of CF infected patients succumb due to respiratory failure brought on by chronic bacterial infection and associated airway inflammation (Lyczak et al., 2002).

In healthy lungs, however, the airways below the first bronchial division remain sterile, because of the existing defence mechanisms that ward off the virus and bacteria present in the air we breathe. Lung defences consist not only of mechanic defences but, also, of cellular immune mechanisms as dendritic cells, neutrophils and macrophages recruited by signalling molecules in the airway surface liquid (ASL) (Wine, 1999). The ASL is a mucus gel and periciliary sol that forms a thin covering layer of the airways, consisting of proteases/antiproteases, oxidants/antioxidants, antibiotics, and antibodies that work together to inactivate or destroy pathogens, without collateral damage to the lungs. Additionally, the ASL is propelled towards the mouth by coordinated ciliary beating which aided by cough helps the mucociliary clearance of the airways. In contrast, in CF most of the airways are infected, thus making it difficult and impossible to clear the infections (Wine, 1999).

The CFRT gene product is an anion channel, member of the ATP binding cassette (ABC) family, which uses ATP hydrolysis energy to change between conducting and non-conducting conformations, but in CF this channel its affected, explaining a wide range of symptoms (Lyczak et al., 2002; Wine, 1999). As an anion channel the CFRT product is important to both absorption and secretion because it allows the flow of anions to in or out of the cells. Nevertheless, in CF the CFRT its defective which results in a loss of CFRT-mediated anion conductance, thus explaining, for example, the high levels of electrolytes, such as chloride, in CF patients sweat. There are, at least, two hypotheses that explain the CFRT role in cystic fibrosis lung disease and both view this anion

channel role in salt absorption to be of importance, indicating that this protein has, at least, two functions those being to conduct Cl^- ions and to regulate epithelial Na^+ channels (EnaC) (Lyczak et al., 2002; Wine, 1999).

Firstly, the high salt hypothesis **(Fig. 3A)** considers that the defective or missing CFRT allows salt levels in the ASL to be similar to those in the plasma **(Fig.3A1)** which interferes with the antibiotics present in the ASL **(Fig.3A2)**. Secondly, the low volume hypothesis states **(Fig.3B)** that, although, CF ASL exhibits plasma-like salt levels **(Fig.3B1)**, the CFRT mutations eliminate the CFRT's inhibition of the EnaC channels, consequently, increasing Na^+ transport, which in turn, increases Cl^- transport and water absorption **(Fig.3B2)**. As stated before the ASL has two components a mucus layer which entraps the pathogens and particles we breathe and the pericilliary liquid that acts as a cell surface coating and helps cilliary beating. In consequence, the volume depletion that happens in CF airways surfaces induces a slower mucus transport and bacterial clearance, and mucus adhesion to the airway surface which results in airway obstruction (Boucher, 2004; Mall et al., 2004). Furthermore, the ASL bactericidal activity is affected in CF because of the high salt concentration which impairs the activity of the bactericidal factors (Smith et al., 1996). Hence, the various CFRT mutations lead to infection and obstruction of the airways caused by the decreased bactericidal activity and accelerated fluid absorption that depletes the ASL volume (Boucher, 2004; Smith et al., 1996; Wine, 1999).

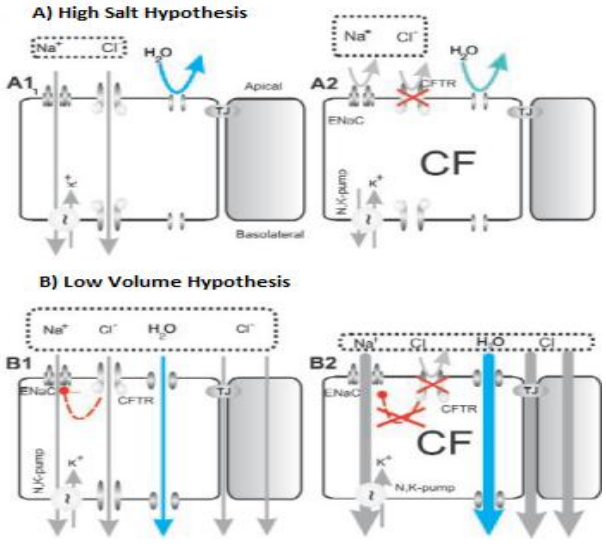


Fig. 3: Two different Hypothesis of how CFRT mutation affects the ASL. **A)** The high salt hypothesis shows that normal ASL has low levels of salt as result of salt absorption **(A1)** In CF **(A2)** salt is poorly absorbed which results in a high salt concentration in ASL that disturbs natural mucosal antibiotics. **B)** The low volume hypothesis proposes that the normal ASL has salt levels approximately equal to plasma **(B1)**. In CF **(B2)** the CFRT inhibition of EnaC channel is lost resulting in an abnormally elevated isotonic fluid that leads to the depletion of the ASL and defective mucociliary clearance (Adapted from Wine, 1999).

1.5 *B. cenocepacia* host-cell interactions

Infection, in cystic fibrosis (CF) patients can be caused by opportunistic pathogens like the members of the *Burkholderia cepacia* complex. In addition, some Bcc strains are associated with heightened transmissibility, poor clinical outcome, and increased risk of developing *cepacia* syndrome, as well, as being associated with bacteraemia in severely ill non-CF patients; thus suggesting tropism of these organisms to injured lungs (Ganesan & Sajjan, 2012). Strains of the ET12 lineage, like *B. cenocepacia* K56-2, preferentially enter CF airway epithelial cells **(Fig.4B)** (Sajjan et al., 2004; Sajjan et al., 2006). So, to survive in an intracellular environment these species evades host defences by employing several mechanisms involved in intracellular survival and replication **(Fig.4C)**.

Firstly, Bcc complex bacteria have the ability to infect host cells, through the development of biofilms **(Fig.4A)** (Schwab et al., 2002). To develop a mature biofilm these bacteria need to form microcolonies which are often found within the mucus layer, on the surface of epithelial cells or deeper in the cell layer, indicating cellular invasion (Sajjan et al., 2004). Normal apical mucus prevents bacteria from reaching the underlying epithelial cells, due to factors that kill and/or prevent replication of bacteria (Sajjan et al., 2004). However, CF mucus traps bacteria but it does not reduce its density or damage, so, trapped bacteria have longer periods to replicate and increases chances of adherence and invasion of the underlying epithelial cells (Sajjan et al., 2004).

Once, these bacteria aggregate and show characteristics of a distinct biofilm the bacteria are able to invade airway epithelial cells (Schwab et al., 2002). Then, after internalized through phagocytosis by both professional phagocytes and epithelial cells, Bcc bacteria, as *B. cenocepacia*, subverts trafficking of young phagossomes (Burns et al., 1996; Martin & Mohr, 2000; Schwab et al., 2002). *B. cenocepacia* either diverts young phagossomes from their normal trafficking or exits from early endossomes to reside in the cytoplasm or enter vesicles. In addition, these bacteria are, also, capable to evade the endocytic pathway, prevent maturation of lysossomes, inhibit the acidification of autophagossomes and to be found in perinuclear area, suggesting that these bacteria traffic and replicate in the endoplasmatic reticulum (ER) (Sajjan et al., 2006).

Besides, invading able to disrupt the actin cytoskeleton and to disrupt or rearrange the intermediate filaments (Sajjan et al., 2004; Schwab et al., 2002; Schwab et al., 2003), as well as, migrate across polarized respiratory epithelium expressing tight junctions (Kim et al., 2005). For example, in order to cause infection, *B. multivorans* promotes the disruptions of actin filaments during epithelial host cell invasion; once the actin network is disrupted biofilm-dependent invasion ceases to be effective, suggesting that this invasion mechanism is dependent on an intact

cytoskeleton (Schwab et al., 2003). Furthermore, CF epithelium infected with *B. cenocepacia* K56-2 showed bacteria between and within cells, despite the presence of mucus. These bacteria were free or surrounded by intermediate filaments, indicating that their movement may involve disruption or rearrangement of the intermediate filaments (Sajjan et al., 2004).

As previously referred, Bcc complex members are able to migrate across the epithelium **(Fig.4D)** (Kim et al., 2005). *B. cenocepacia* infects polarized respiratory epithelium by compromising epithelial integrity through the disruption of intracellular tight junctions, which, results in a steady decline of the transepithelial electrical resistance. In fact, cell layers infected with *B. cenocepacia* revealed its colocalization with occludin which appeared to be disrupted and translocated to the cytoplasm. Although, infected cells show disrupted occluding localization, tight junction organization was not disturbed indicating the absence of a secreted toxin capable of tight junction disruptiom, as seen in other pathogens as *C. difficile* (Kim et al., 2005; Sears et al., 2000). In contrast, to such pathogens these bacteria do not cause disruption of the cell layer due to cell death or injury, instead *B. cenocepacia* is capable of occludin dephosphorylation and its dislocation from focal points along the tight junctions' axis. Therefore, leading to the disruption of tight junction integrity, that in turn allows the migration of bacteria through the epithelium (Kim et al., 2005).

Moreover, *B. cenocepacia* infection produces distinct responses from healthy well differentiated airway cell cultures and CF cell cultures. In normal cell cultures with apical mucus layer *B. cenocepacia* bacteria are not able to decrease transephitelial resistance, however, in CF cell cultures these bacteria's infection caused the decrease of transepithelial resistance and increase of permeability; especially when the apical mucus layer was removed (Sajjan et al., 2004).

Additionally, infected CF cells, whether with depleted mucus layer or not, demonstrated an increased interleukin-8 (IL-8) expression. Also, only Bcc complex strains that persisted on the epithelial cells, elicited a significant IL-8 response, implying that these bacteria have to bind and/or invade the underlying epithelial cells to promote IL-8 release (Sajjan et al., 2004).

Finally, strains as *B. cenocepacia* J2315 and *B. cepacia* J2540 are not only able to invade epithelial cells, but, also, capable of macrophage invasion. Nevertheless, while *B. cepacia* J2540 was not able to survive and replicate intracellularly. *B. cenocepacia* J2315 was able to survive and increase its numbers located intercellularly 24 hours after infection (Martin & Mohr, 2000).

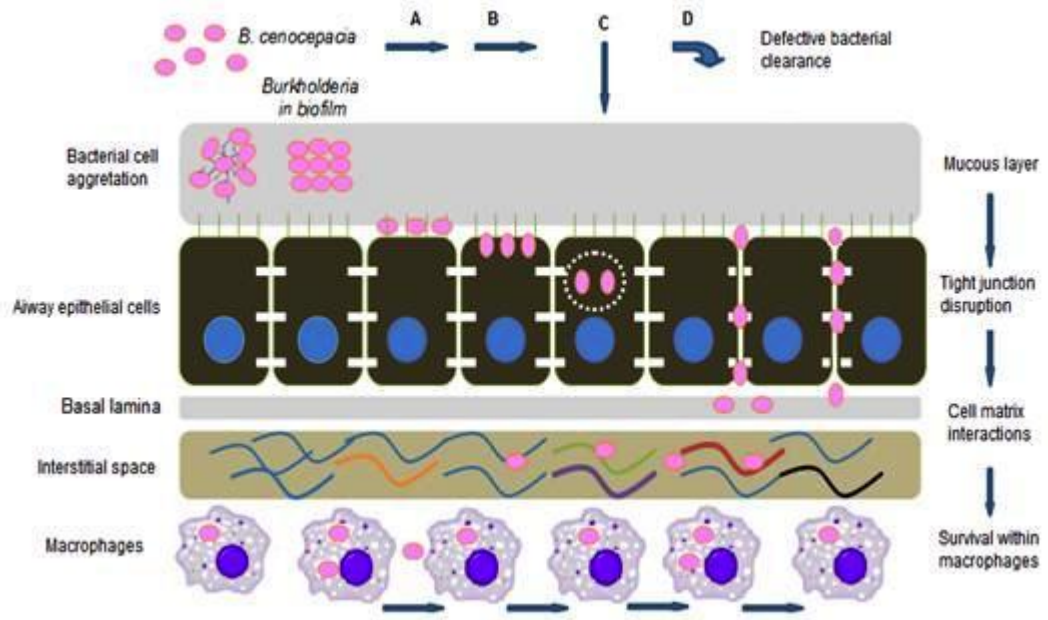


Fig. 4: *Burkholderia sp.* mechanism of airway epithelia infection. **A**) Bacterial attachment to the cell apical mucus and biofilm development. **B)** Airway epithelial cell invasion. **C)** Intracellular survival and replication. **D)** Bacterial migration across the epithelium by means of occludin desphosphorylation and its dislocation from focal points of the tight junction axis

Moreover, once chronic colonization, in CF patients, is settled the eradication of these organisms is unlikely. Therefore, several combinations of antibiotic and non-antibiotic compounds are currently being tested against *Burkholderia* and *Pseudomonas. Burkholderia* are often difficult to eradicate as they usually are intrinsically resistant to multiple antibiotics (Moss, 1995; Govan & Deretic; 1996, Ramsey, 1996). Nevertheless, *Pseudomonas* infections often begin by resembling bronchitis that is generally cleared by antibiotics, but, later replaced by more difficult to eradicate chronic colonizers of the airways (Burns et al., 2001). The usual chemotherapeutic strategies apply one or more classes of antibiotics in which the most common pairing is a β-lactam with an amino glycoside for which increased efficacy has been demonstrated in CF patients (reviewed in George et al., 2009). Furthermore, a *in vitro* study demonstrated excellent synergy of polyethylenimine with β-lactams, cephalosporins, novobiocin, or chloramphenicol, which resulted in diminished minimal inhibitory concentrations (MICs) to the these antibiotics against *P. aeruginosa.* On the other hand the bismuth-thiols showed *in vitro s*ynergy with tobramycin against the Bcc complex, as well as, possessing advantages against biofilm activity and micromolar MICs (Veloira et al., 2003).

1.6 *Burkholderia cepacia* complex genome

Burkholderia species have some of the largest and most complex bacterial genomes studied. All *Burkholderia* species possess large genomes ranging from 6 to 9 Mb and it's this huge genetic capacity that grounds out their great versatility both in natural environments and in disease. Bcc species, also, separate its DNA into two or more chromosomal replicons which may add greater flexibility in the gain, loss and expression of genes (Mahenthiralingam et al., 2008).

As *B. cenocepacia* is the most clinically important member of the *B. cepacia* complex that causes infections in cystic fibrosis patients. The genome of *B. cenocepacia* J2315, a multidrug resistant clinical isolated of the ET12 lineage, has been studied to provide insight into the success of this strain as an opportunistic pathogen. The complete genome of *B. cenocepacia* J2315 consists of 3 circular chromosomes (3.9Kb, 3.2Kb and 876bp) and a plasmid (92,661bp) **(Tab. 2)** (Holden et al., 2009). Analysis of all 4 replicons revealed several encoded initial coding sequences (CDS) of which 126 are pseudogenes or partial genes. On chromosome 1 diverse functions are encoded those range from Rnase E, ribossomal protein, fatty acid/phospholipid synthesis proteins to elongation factor P, while chromosome 2 and 3 encode greater proportion of accessory functions (Holden et al., 2009).

Comparative genomics of the J2315 genome with Bcc and other *Burkholderia* genomes showed that the level of orthology identified correlated with their taxonomic relatedness, as the largest number of orthologous coding sequences (CDSs) were identified in the Bcc species, 63 to 78% of total CDSs. In addition, the level of orthology is the greatest on chromosome 1 with chromosome 2 and 3 being progressively more divergent, despite the distribution of orthologs on the different replicons being similar to that seen in various *Burkholderia* species. Furthermore, pairwise alignments demonstrated that chromosome 1 displays the highest level of conservation, followed by the second and third chromosomes which showed lower levels of overall conservation and no conservation of gene order, respectively (Holden et al., 2009).

Tab. 2: General features of the *Burkholderia cenocepacia* J2315 genome (Holden et al; 2009)

Properties	Chromosome 1	Chromosome 2	Chromosome 3	Plasmid	Total
Size (bp)	3,870,082	3,217,062	875,98	92,66	8,055,782
G+C content (%)	66.7	67.9	67.9	62.6	66.9
Number of CDSs	3,54	2,85	776	99	7,26
Coding (%)	86.1	86.4	86.0	78.6	85.9
Average gene length (bp)	958	985	986	763	970
rRNA	4 (16S-23S-5S)	1 (16S-23S-5S)	1 (16S-23S-5S)	0	6 (16S-23S-5S)
tRNA	66	6	2	0	74
Miscellaneous RNA	15	3	2	1	21
Pseudogenes and partial genes	56	41	23	6	126
IS elements	51	16	11	1	79

Horizontal transfer of DNA has been seen as an important factor in the success of *B. cenocepacia* in disease and spread between patients. In the studied J2315 genome 14 regions have been identified as putative genomic islands. For example, the *cci* island, a 44kb region, has been shown to be involved in infection as it has attributed functions like antibiotic resistance and a quorum sensing system (Holden et al., 2009).

Moreover, the annotation of close repeats in the *B. cenocepacia* J2315 sequence revealed 1056 repeats, that vary from a minimum of 10bp to a maximum of 50 068bp, from which 422 are close DNA repeats, among these repeats 45 genes are potentially involved in virulence (Mil-Homens et al., 2010). 13 of these genes were included in the putative antigen class and the product of four genes was found to vary in length, due differences in the number of tandem repeats. In addition, the putative antigens BCAM0219 and BCAM0223, annotated as trimeric autotransporter adhesins (TAAs) were absent from other genomes (Mil-Homens et al., 2010).

The full transcriptomic analysis of *B. cenocepacia* demonstrated that this pathogen alters the expression of 10% of the 7176 genes within its genome when it grows in CF sputum (Drevinek et al., 2008). Among the genes with altered expression were not only genes with putative functions in antimicrobial resistance, iron uptake, protection against reactive oxygen and nitrogen species, secretion and motility, but also, novel uncharacterized genes which included a transmembrane ferric reductase that's possibly implicated in iron metabolism, a protease that may be important in host tissue destruction, an organic hydroperoxide resistance gene, an oxidoreductase and a nitrite/sulfite reductase that may be relevant in resistance to the host

defences (Drevinek et al., 2008). This transcriptomic study, also, demonstrated that many of the gene pathways activated by *B. cenocepacia* in response to growth in CF sputum correlated with virulence mechanisms, as for example, antibiotic efflux and degradation (Drevinek et al., 2008).

Furthermore, the pilus associated adhesin gene, *adhA,* was found to be activated in sputum suggesting its importance for bacterial growth, however, in contrast, there was a lack of activation of the structural components of cable pilus operon of *B. cenocepacia* J2315 (Drevinek et al., 2008). In addition, genes on the *cci* island were not upregulated and the quorum-sensing systems described in *B. cenocepacia* did not demonstrate differences in expression which suggested that these virulence factors are not important in the modelled early stages of infection. Nevertheless, the upregulation of flagellar apparatus genes was detected suggesting that motility helps *B. cenocepacia* bacteria to maintain a more invasive phenotype during infection (Drevinek et al., 2008).

1.7 Virulence determinants

Although the pathogenicity of these bacteria is not fully understood a vast range of virulence determinants have already been studied. Those include extracellular and cell surface polysaccharides, nutrient acquisition and metabolic pathways, resistance to antimicrobials and proteins.

1.7.1 Quorum sensing

Bacteria use quorum sensing (QS) systems as a means to quickly adapt to a given environment, as the QS systems are often responsible for siderophore, protease and lipase production, and biofilm formation (de Kievit & Iglewski., 2000). In the Bcc complex the most widely distributed QS system is the *cepIR* system, initially, described in *B. cenocepacia,* which regulates extracellular virulence factors as zinc metalloproteases and biofilm formation that contribute to lung injury (Sokol et al., 2003).

1.7.2 Siderophores

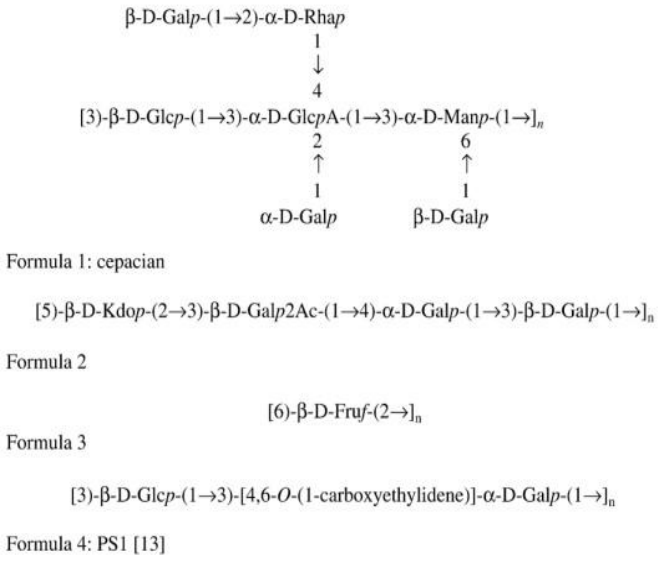
CF patients lungs are iron restricted environments for prospective pathogens as the Bcc species. In order to be able to cause infection *B. cepacia* complex members possess efficient iron uptake mechanisms. These bacteria display up to four types of siderophore – ornibactin,the most prevalent (Darling et al., 1998), pyochelin, cepabactin and cepaciachelin – that employ nearly all iron-binding groups present in most siderophores (Thomas, 2007). Moreover *B. cenocepacia* ornibactin mutants showed attenuated virulence against both *C. elegans* and *G. mellonella* (Uehlinger et al., 2009) and a mutation in the ferric-ornibactin receptor severely hindered the growth of *B. cenocepacia* K56-2 (Visser et al., 2004).

1.7.3 Lipopolysaccharides

Lipopolysaccharides (LPS) are glycolipids, capable of inducing a strong immune response which contributes to host cell damage. In *B. cenocepacia* the LPS plays an important role in virulence, since mutants on genes concerning LPS biosynthesis have damaged virulence in rat agar bead model, *C. elegans* and *G. mellonella* (Loutet et al., 2006; Hunt et al., 2004., Uehlinger et al., 2009). The *B.cenocepacia* species LPS is, as well, able to affect the level of phagocytosis and induce a strong cytokine inflammatory response that varies within the *B. cenocepacia* strain (Bamford et al., 2007; De Soyza et al., 2004; Saldías et al., 2009).

1.7.4 Exopolysaccharides

Exopolysaccharides (EPS) usually have two functions, to form a barrier around bacterial cells or colonies and to interfere with the host defences through their chemical characteristics (Chiarini et al., 2004). Most of Bcc strains including *B. cenocepacia,* have been shown to mostly produce one type of EPS named cepacian, which is often produced along with other polysaccharides species **(Fig.5)** (Chiarini et al., 2004; Herasimenka et al; 2007, Zlosnik et al; 2008). *B. cenocepacia* is capable to exhibit both mucoid and non-mucoid phenotypes, which are related with the high and low EPS production, respectively (Cérantola et al., 2000; Zlosnik et al., 2008). The mucoid phenotype is associated with delayed lung clearance in a mouse model, and interferes with the binding and/or with phagocytosis by neutrophils and mononuclear phagocytes, development of microcolonies, establishment of thick and mature biofilms *in vitro* and substratum adherence (Conway et al., 2004; Cunha et al., 2004). Furthermore, non-mucoid *B.cenocepacia* bacteria are associated with increased disease severity while the mucoid phenotype can be associated with persistance in CF airways (Cunha et al., 2004, Zlosnik et al., 2008).



Formula 1: cepacian

[5)-β-D-Kdop-(2→3)-β-D-Galp2Ac-(1→4)-α-D-Galp-(1→3)-β-D-Galp-(1→]n

Formula 2

[6)-β-D-Frufp-(2→]n

Formula 3

[3)-β-D-Glcp-(1→3)-[4,6-O-(1-carboxyethylidene)]-α-D-Galp-(1→]n

Formula 4: PS1 [13]

Fig. 5: Chemical structure of the EPS produced by Bcc (Adapted from Herasimenka et al., 2007).

1.7.5 Flagella

B. cepacia is a motile organism, and motility is mediated by polar flagella. Flagella in *B.cepacia* affects both swarm capacity and invasion of host cells, as flagellum-mediated motility is necessary for the establishment of contact with host cells and, then, subsequent bacterial entry; therefore contributing to the adherence of bacteria to epithelial airways, invasion of deeper tissue,

and penetration of the abundant mucus layer in CF patients that covers the airways, due to inefficient mucociliary clearance (Tomich et al., 2002).

Furthermore, *B. cenocepacia* flagella contribute for both infectivity and inflammation, as it contributes to the virulence in an *in vivo* infection model, and induction IL-8 secretion through interaction with TLR5 may contribute to lung damage (Urban et al., 2004).

1.7.6 Pili and the associated 22KDa adhesin

A study demonstrated that *B. cepacia* isolates carrying the *cblA* gene, and,the 22KDa adhesin, with cable pili phenotype are capable to adhere to CF damaged lung tissue, by binding to cytokeratin 13 (CK) which is enriched in CF lung tissue (Sajjan et al., 2000). The 22KDa adhesin is sufficient for the binding, adherence and the transmigration across squamous epithelium of *B. cenocepacia* bacteria but it alone does not account for the optimal binding to the CK13 receptor, as, both cable pili and the adhesin are necessary for optimal binding and transmigration through the epithelia (Urban et al., 2005) Moreover, both the 22KDa adhesin and the cable pili contribute to IL-8 response, inflammation and persistence of *B. cenocepacia in vivo* and both account for spread of infection through the airways (Goldberg et al., 2011).

1.7.7 Secreted proteins

The *Burkholderia* species express at least two proteases, the ZmpA and ZmpB zinc metalloproteases, which are able to directly degrade important host tissues components like collagen and fibronectin as well as cleave immune response agents as immunoglobulins (e.g. IgA and IgG), therefore, leading to lung tissue damage and impairment of immune host defences, that in turn contribute to the virulence of these bacteria (Corbett et al., 2003; Kooi et al., 2005; Kooi et al., 2006). Furthermore, different secretion types that support pathogenicity, virulence and persistence of diverse species of Bcc bacteria have been described, as for example type III, VI and IV secretions systems (Sajjan et al., 2008, Scharwz et al., 2010, Stevens et al., 2004 ; Schell et al., 2007; Ulrich et al., 2004).

1.7.8 Antimicrobial resistance

Bcc bacterial infections are difficult to contain because many strains are multidrug resistant either because of intrinsic or acquired mechanisms being the usually administered therapy frequently composed of two or three different agents, even though the optimal had not yet been found (Avgeri et al; 2009, Sass et al;2011) *B. cenocepacia* exposure to several antibiotics revealed a wide range of resistance mechanisms as for example, beta-lactamases, phosphohydrolases, efflux

pumps and phenylacetic acid pathways that could be used as potential therapeutic targets. (Sass et al., 2011).

1.7.9 Biofilms

As already referred in the chapter Cystic Fibrosis and *Burkholderia spp*. Infection these bacteria ability to develop biofilms plays an important role in its capacity to cause infection. In addition, *B. cenocepacia* and *B. multivorans* besides being the most abundant strains isolated from CF patients are, also, the strains that form the most considerable amount of biofilm, therefore, providing these strains with a possible advantage against the host antimicrobial defences (Conway et al., 2002). Although, there have been instances where studied showed there's no correlation between biofilm development and antibiotic resistance, and, in contrast, others studies that demonstrated the increase of antibiotic resistance in biofilms (Peters et al., 2009; Van Acker et al., 2010).

1.7.10 Resistance to reactive oxygen species (ROS)

All bacteria, including Bcc strains, have mechanisms meant to cope with ROS created either from their own endogenous metabolism or from their environment. Since, CF patients suffer from chronic lung inflammation their sputum is a source of harmful ROS compounds (Drevinek et al., 2008). Therefore, when these bacteria are subjected to stress there are different genes activated in response – the *ohr* gene, *asmC* gene – and the *hfq* gene, which is, also, important for stress tolerance of *B.cepacia* (Drevinek et al., 2008; Sousa et al., 2010).

1.8 Trimeric autotransporter adhesins

Trimeric autotransporter adhesins (TAAs) **(Fig.6)** are multimeric proteins found only in gram-negative bacteria. These proteins are important virulence determinants for *Burkholderia* strains being responsible for not only adherence but, also, biofilm formation, invasion and survival within eukaryotic cells (Mil-Homens & Fialho, 2011). Therefore, the trimeric autotransporter adhesins are an important intervenient in the adhesion process to extracellular matrix proteins (ECM) and to host cells (Linke et al., 2006). TAAs are multi-domain outer membrane proteins organized in modules which in this case means that there's a membrane anchored C-terminal domain that forms a trimeric 12 stranded beta-barrel pore that allows the translocation of the passenger domain into the extracellular space, using a type V secretion system (Cotter et al., 2005). Also, the trimerization of these proteins is extremely crucial for their function and translocation as it provides TAAs with the stability needed for multivalent interactions (Lehr et al., 2010).

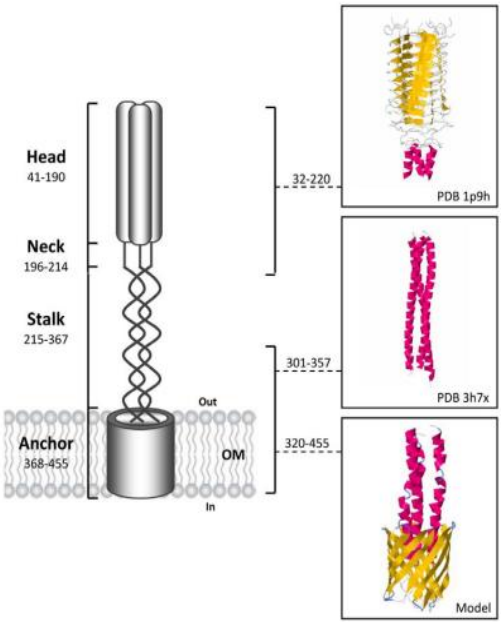


Fig. 6: Trimeric autotransporter adhesin schematic representation (Adapted from Mil-Homens & Fialho., 2011)**.**

The analysis of the *B. cenocepacia* genome revealed 74 TAA putative encoding sequences showing the large number of TAAs that Bcc genomes possess reflecting their large multireplicon genomes that may have been acquired through insertion of transposable elements and bacteriophages or through horizontal transfer of DNA fragments (Mil-Homens & Fialho, 2011). Additionally, the high number of TAAs found in these genomes might suggest their high genome plasticity and their ability to colonize and adhere to their hosts whether human or environmental,

even though density and number of TAAs varies among the Bcc members. Furthermore, the phylogenetic analysis and sequence alignment of the TAAs from the Bcc genomes showed, that the domain architecture is conserved, being the C-terminal translocator domain highly conserved (Mil-Homens & Fialho, 2011).

Seven TAAs were annotated in the genome of *B. cenocepacia* J2315, five were identified on chromosome 2 – BCAM0219, BCAM0223, BCAM0224, BCAM2418 and BCAM1115- and, other two on chromosome 3 – BCAS0236 and BCAS0335- confirming the findings of Holden et al. 2009 in which chromosome 2 and 3 present the most of the virulence genes instead of chromosome 1 where core functions are located on (Mil-Homens & Fialho, 2011). In addition, the adhesins BCAM0219 and BCAM0223 are part of a putative adhesin cluster **(Fig.7)** found only on *B.cenocepacia* strains of the ET12 lineage. The mentioned adhesins are contained within the mentioned cluster as three adhesins - BCAM0219, 0223, 0224 - one outer membrane protein- BCAM0220 - one sensor histidine kinase - BCAM0218 - and, lastly, two regulators - BCAM0221 and BCAM0222 (Mil-Homens et al, 2010). Moreover, it was suggested that, the co-transcribed BCAM0219 and BCAM0220 genes, and the TAAs genes BCAM0223 and BCAM0224 are regulated by the two present regulators while the sensor histidine kinase is responsible for sensing stimuli (Mil-Homens et al, 2010). Furthermore, this adhesin cluster is placed upstream of the *cci* pathogenicity island, identified in *B. cepacia* J2315, which includes BCESM the epidemiological marker for virulent strains. The BCAM0224 gene was found to be unique to ET12 lineage *B. cenocepacia* (Mil-Homens et al, 2010).

Fig. 7: Genetic organization of the putative adhesin cluster. The numbers 218,219,220,221,222,223,224 and 225 refer to the BCAM0219*, 0223, 0224* and *0225* genes, respectively. The *B. cepacia cci* island is also shown (Mil-Homens et al., 2010).

As stated before, the TAAs are important to the virulence and adhesion of these bacteria to host cells. The BCAM0224 adhesin, was demonstrated to be able to bind to type I collagen, an important and abundant component of the extracellular matrix, and to play a role in the infection and virulence of *B. cenocepacia* infected CF patients. As, in fact, a BCAM0224 mutant displayed a marked decrease in virulence when compared with the wild type which implies the association of

TAAs with the proper attachment to host cells (Mil-Homens et al., 2010). In addition, this adhesins may play an important role in the development of bacteria aggregates because the BCAM0224 mutation made *B. cenocepacia* incapable to do so, therefore, implying that this adhesin endogenous production is needed for cell-to-cell adhesion. TAAs are structurally rigid proteins as their 3D structure shows, in order to provide stability, three intrinsically connected trimers and when subjected to external force TAAs act as a spring which is consistent with their structure (El-Kirat-Chatel et al., 2013). Additionally, it was demonstrated that the BCAM0224 TAA is capable to bind itself to specific receptors in the membrane surface of airway epithelial cells and form important invasion membrane tethers (El-Kirat-Chatel et al., 2013).

Furthermore, a functional study of the trimeric autotransporter adhesin BCAM0223 as further demonstrated the necessary role of TAAs as they influence, not only, adherence to epithelial cells, but, also, serum resistance and hemagglutination (Mil-Homens & Fialho., 2012). The BCAM0223 mutant when compared with the wild strain showed marked decrease of hemagglutination, as well as, impaired capability to adhere to extracellular matrix proteins, as vitronectin, and to form biofilms in polysterene surfaces pointing that this TAA might be needed for, not only, biofilm development, but, for persisting infection. This study, also, demonstrated that the classical complement pathway is necessary for the killing of the BCAM0223 mutant. As it, likewise, showed, whether the cells were afflicted by CF or not, the impaired ability of this mutant strain to adhere to respiratory epithelial cells, in comparison with the wild-type strain. Besides, the adhesion decrease in the mutant implies that on one hand, non-CF cells expose a low level of BCAM0223 receptors, while, on the other hand, the mutant strains had a better invasion capacity than the wild-type. It is still not known why the BCAM0223 mutant is more efficient in invading host cells but one possible explanation is that in the absence of one of the TAAs, other multi-functional TAAs and other adhesins, as well as, other cell surface components are capable to improve the efficiency of alternative invasion factors. Moreover, the role of the BCAM0223 adhesin was, also, evaluated *in vivo* in *Galleria mellonella,* and, although, moderated, the lack of the BCAM0223 adhesin diminishes the virulence of *B. cenocepacia* K56-2. Lastly, it is conceivable to suggest that this adhesin contributes to the virulence of *B. cenocepacia* (Mil-Homens & Fialho., 2012).

1.8.1 TAAs biological traits

As explained before, TAAs mainly have an adhesive function nevertheless they can usually accumulate other functions implicated in many other virulence roles (**Tab.3)**. This following chapter

will describe the biological functions of some of these proteins.

Tab. 3: Examples of characterized TAAs

Organism	Protein	Functions	References
Y. enterocolitica *Y. pseudotuberculosis*	YadA	ECM binding. Autoagglutination Serum and phagocytosis resistance. Binding to epithelial cells and neutrophils. Invasion of epithelial cells.	(Eitel & Dersch, 2002; El Tahir et al., 2000)
M .catarrhalis	UspA1 UspA2	ECM and epithelial cell binding. Serum resistance. Bio film formation.	(Holm et al., 2003; Pearson et al.,2002; Pearson et al.,2006 Riesbeck et al., 2006;)
	MID/Hag	IgD binding. Adhesion to lung and middle ear epithelial cells Inhibitory effect on biofilm formation Hemagglutination, Autoagglutination.	
H. influenzae	Hia Hsf	Epithelial cells binding.	(Barenkamp & Geme, 1996)
S. enterica	SadA	Epithelial cells binding. Biofilm formation. Autoaggregation.	(Raghunathan et al., 2011)

1.8.1.1 The *Yersinia* YadA adhesin

Several species from the *Yersinia* genus are considered pathogens, *Yersinia enterolitica* and *Yersinia pseudotuberculosis* are food-borne pathogens causative of gastrointestinal infections, while *Yersinia pestis* is the agent of the Plague, a zoonotic disease that mainly affects small animals (Chain et al., 2004; Gu et al., 2007).

The YadA adhesin is a major virulence determinant of the *Y. enterolitica* species and it belongs to the TAAs family, which is enconded on the pYV virulence plasmid (el Tahir & Skurnik., 2001; Linke et al., 2006; Zaleska et al., 1985). Many functions as causing infection, serum resistance, autoagglutination, phagocytosis resistance, invasion and, lastly, adhesion to the ECM components, have already been attributed to this adhesin (Balligand et al., 1985; Emody et al., 1989; Heesemann et al 1987; Shurnik et al., 1984; Tertti et al., 1992). The expression of the YadA protein is regulated and induced by a temperature of more than 37ºC (Shurnik & Toivanen, 1992). For the species *Y. enterolitica* this adhesin is most important as it binds to various types of fibrillar collagen, which include types I, II, III, V, as well as the network forming collagen type IV (Schulze-Koops et al.,1992), but, in contrast, for *Y. pseudotuberculosis*, it binds to fibronectin instead of collagen and

laminin (Heise & Dersch, 2006). Furthermore, the YadA adhesin is, also, able to mediate the bacterial adhesion to different types of cells as epithelial cells, neutrophils and macrophages (Leo & Shurnik., 2011), in addition, to its ability to block the three pathways of the complement (Lambris et al., 2008), despite it being only important to *Y. enterolitica* persistence and not to *Y. pseudotuberculosis* (El Tahir & Shurnik., 2001). Lastly, the YadA adhesin ability to bind to collagen is crucial to the virulence of *Y. enterolitica* as its absence causes the avirulence of these bacteria in mice (Roggenkamp et al., 1995).

1.8.1.2 The *Moraxella catarrhalis* adhesins

Moraxella catarrhalis is a gram negative bacterium commonly found in the lower respiratory tract where it causes severe infections and aggravation of obstructive pulmonary disease (Verduin et al., 2002). TAAs in this species are, also, responsible for their ability to adhere to human epithelial cells (Lafontaine et al., 2000), as the adhesins UspA1 and UspA2 bind to ECM components like fibronectin and laminin, and additionally UspA2 is, as well, capable to bind to vitronectin (Hill et al., 2012; Tan et al., 2005; Tan et al., 2006). Moreover, the UspA2 protein is, also, associated with serum resistance and the ability of these bacteria to interfere with the complement pathway and the UspA1 protein is able to bind to the carcinoembryonic antigen related cell adhesion molecule (CEACAM) which is widely distributed on respiratory tract cells, and is involved in biofilm formation (Hill et al., 2012, Hill & Virji., 2003).

Besides, the UspA adhesins *Moraxella catarrhalis*, also, expresses the MID IgD-binding protein which is a TAA, as well (Pearson et al., 2002). The MID adhesin has the ability to agglutinate erythrocytes and to bind to type II alveolar epithelial cells, mediate binding to type IV collagen, activate IgD^+ B cells and provide *Moraxella catarrhalis* the ability to avoid interaction with host B cells through the secretion of antigen bearing outer membrane vesicles (OMV) (Bullard et al., 2007; Forsgren et al., 2003; Jendholm et al., 2008; Vidakovics et al., 2010). Additionally, *M. catarrhalis* devoid of MID adhesin were more efficiently cleared from mice lungs than wild type bacteria (Forsgren et al.,2004).

1.8.1.3 The *Haemophilus influenzae* Hia and Hsf adhesins

In the species *H.influenzae* two homologous TAAs were found - the Hia and the Hsf - that promote the adherence to respiratory epithelial cells (Spahich & St. Geme III, 2011). The Hsf adhesin has two binding domains- the HsfBD1 and the HsfBD2- that exhibit significant homology

with the Hia high affinity domain - HiaBD1 - and mediate adherence through a conserved acidic binding pocket. Furthermore, the Hsf adhesin has a third domain, the HsfBD3, which lacks the acidic binding pocket and the adhesive ability in Chang epithelial cells, despite, also, sharing similarities with the HiaBD1. Therefore, this last domain might have an important role in stabilizing Hsf fibers and facilitating Hsf-mediated adherence in a context of polysaccharide capsule (Cotter et al., 2005).

The other TAA expressed by *H. influenzae* is the Hia adhesin which promotes the adherence to several human epithelial cell lines. The Hia adhesin passenger domain contains two different binding pockets, which with different affinities interact with the same host cell receptor. Additionally, both domains were shown to be essential optimal bacterial adherence (Laarman et al., 2002).

1.8.1.4 The *Salmonella enterica* SadA adhesin

The *S. enterica* SadA TAA is involved in promoting epithelial cell binding autoaggregation and biolfilm formation (Raghunathan et al., 2011). Nevertheless, it was found by further investigation that directly upstream to the adhesin gene, a small lipoprotein was predicted. This trimeric lipoprotein was named SadB and it was demonstrated to be co-expressed and directly influence the SadA TAA. The lipoprotein SadB was shown to be located in the inner membrane of Salmonella spp. and its absence reduced the amount of surface expressed SadA, as well as, its resistance to proteinase K implying that SadB is involved on the folding and exporting of the SadA TAA. Furthermore, the presence of this lipoprotein is required for the surface expression of SadA as the induced expression of both proteins showed higher signal levels of SadA than strains expressing only the *sadA* gene. In addition, the expression of both SadA and SadB, but not the expression of SadA alone, contributed to the development of SadA positive cells clusters (Grin et al.,2013)

1.9 Lipid rafts in cell interactions

CF airways epithelial cells susceptibility to infection may arise due to a number of alterations, which provide receptors for the pathogens to adhere to, as , for example, CF cells show both increased sulfation of high molecular mass glycoproteins, fucosylation and decreased sialylation (Rhim et al., 2000; Xia et al., 2005). In addition, CF cells have a higher ratio of asialylated ganglioside 1 (aGM1) to sialylated ganglioside 1 GM1, and, also, higher expression of surface aGM1 (Saiman & Prince, 1993). This is important because pathogens as *P. aeruginosa*, the most common species in CF infected airways, and Bcc species have been described to bind to asialylated glycolipids (Krivan et al., 1988; Saiman & Prince, 1993), as well as, to lipids receptors (Sylvester et al., 1996). The studies by Krivan et al and Sylvester et al, show contrasting results as the former demonstrated that Bcc strains bind to GalNAcbeta1-4Gal sequences containing glycosphingolipids which include aGM1 and aGM2, and did not bind to the GM1 and GM2 globosides, while, the later study indicated *B. cenocepacia* as able to bind to globotriosylceramides (Krivan et al., 1988; Sylvester et al., 1996).

Several pathogens interact with lipid rafts in order to entry host cells, lipid rafts can be found in eukaryotic cells namely in both their plasma and endossomal membranes and are composed of cholesterol, glycosphingolipids, and glycosylphosphatidylinositol (GPI) anchored proteins **(Fig.8B)** (Rosenberger, Brummel & Finlay., 2000, reviewed in Zaas et al., 2005). Lipid rafts exhibit many functions as polarized secretion, membrane transport, transcytosis across epithelial monolayers and the generation of cell polarity, as well as, playing an important role in signal traduction through their enrichment with many signalling molecules, as for instance, tyrosine kinases and lipid signalling intermediates (Rosenberger, Brummel & Finlay, 2000). Furthermore, a growing number of pathogens admit the use of lipid rafts to gain entry into to host cells or the use of clustered lipid rafts which are assemblies of a number of lipid rafts, thought to be included in macromolecular transport. Pathogens that use clustered lipid rafts as a mean of endocytosis may be able to evade fusion with traditional lysossomes and, therefore, the contents can target a range of different cell compartments, which may help the spread of pathogens and their survival within host cells **(Fig.8A)** (reviewed in Zaas et al., 2005). Lipid rafts besides being important in the binding of toxins are, also, important in the adhesion and the binding of bacteria themselves. It was possible to assume the involvement of lipid rafts because of the observation, in bacterial entry sites, of proteins and raft associated lipids, in addition, to the need of cholesterol presence for bacterial invasion (Lafont & van der Goot, 2005).

P. aeruginosa is among the wide range of bacterial pathogens that utilize lipid rafts as receptors, in which, is possible to find, for example, *Escherichia coli,* that was the first pathogen known to invade host cells via lipid rafts and *Brucella abortus,* which is the bacteria responsible for the most frequent zoonosis Brucellosis (Zaas et al., 2005). A study showed that lipid rafts are necessary for the uptake of *P. aeruginosa* bacteria, and to the location of CFRT to lipid rafts at sites of contact with epithelial cells during the initial interaction. This study, also, indicated, that cyclodextrin, a cholesterol depleting drug, is able to diminish the amount of CFRT found in lipid rafts. Furthermore, cyclodextrin treatment of cells demonstrated reduced uptake of bacteria in both cells expressing wild type CFRT and the ΔF508 mutation. In addition, the internalization levels of *P. aeruginosa* were further reduced by cyclodextrin treatment in cells expressing only ΔF508 mutation in the absence of cyclodextrin. Therefore, these results imply, not only the requirement of cell surface CFRT, but, also, lipid rafts for the internalization of *P. aeruginosa* by epithelial cells (Kowalski et al., 2004). Moreover, these bacteria are, as well, capable to invade the alveolar space and adhere to and invade type I pneumocytes. *P. aeruginosa* showed inhibited invasion of pneumocytes with low doses of ciclodextrin, nystatin and filipin, but, in contrast, adherence to the pneumocytes was not affected by these cholesterol disruption drugs. Additionally, it was shown that not only the integrity of lipid rafts was necessary for *P. aeruginosa* endocytosis , but also, the tyrosine phosphorylation of specific lipid raft proteins as caveolin 2; plus, these bacteria might use this pathway of endocytosis in order to survive within a protected intracellular environment (Zaas et al., 2005a).

Brucella bacteria can avoid the macrophages bactericidal activity after their first contact with host cells and its infection is as well related with lipid rafts. In *Brucella* infection lipid-raft associated molecules as GPI anchored proteins, cholesterol and GM1 gangliosides are found macropinossomes containing *Brucella* bacteria from which the lysosomal glycoprotein LAMP-1 and the host-cell transmembrane protein CD44 were excluded (Jimenez de Bagues et al., 2005; Watarai et al., 2002, Vieira et al., 2010). Additionally, the interaction between the heat-shock protein 60 and PrP^{C} , a lipid-raft associated molecule, on macrophages contributes to the establishment of infection (Watarai et al., 2002, 2004).

Other pathogen that needs the contribution of lipid rafts to establish infection is *N. meningitidis*, that either causes a rapid evolving form of septicaemia or meningitis. Again, the use of a lipid depletion drug rendered *N. meningitidis* microcolonies sensitive to mechanical stress as they were found to be less tightly associated and more easily disrupted; only leaving a monolayer of bacteria in contact with the cells surface. Lastly, the disruption of this lipid microdomain, also,

affected the development of bacteria-induced cellular projections that surround the diplococci microcolonies; as treated endothelial showed reduction in these bacteria-induced cellular projections and cholesterol repletion allowed their development. So, the membrane reorganization brought on by *N. meningitidis* is dependent on the presence of membrane cholesterol for the integrity of lipid rafts, as well as, for the presence of the cellular projections that are relevant for the cohesion of microcolonies (Mikaty et al., 2009).

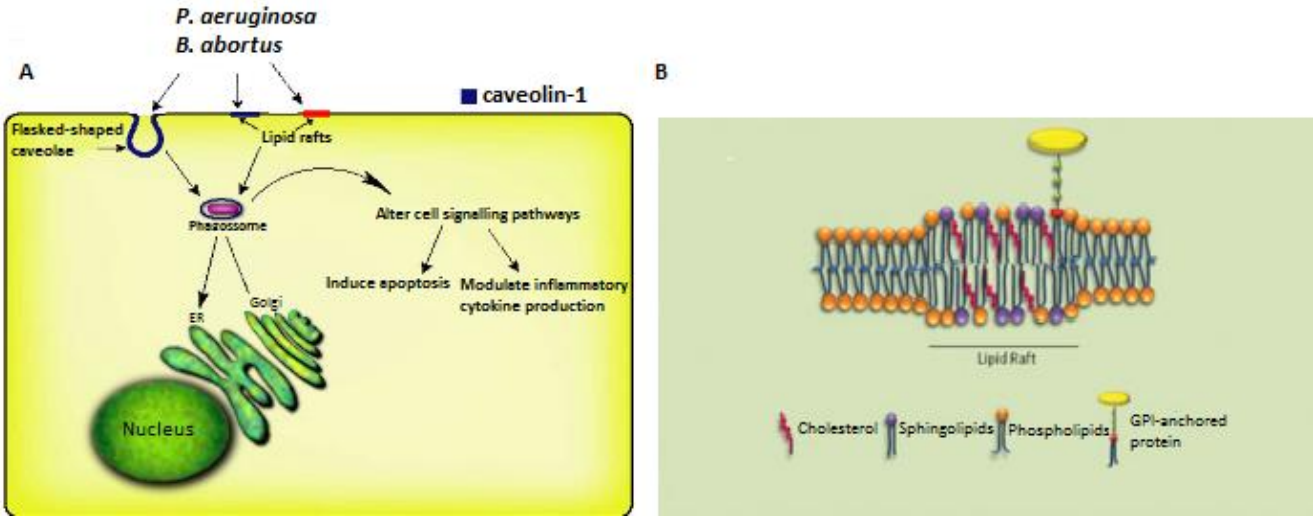


Fig. 8: Schematic diagram showing the role of lipid rafts and caveolae in *P. aeruginosa* and *B. abortus* pathogenesis **(A)** and a classical lipid raft scheme **(B)** where membrane microdomains are enriched with cholesterol, sphingolipids and, also, possess associated proteins such as GPI- anchored proteins (Adapted from Vieira et al., 2005; Adapted from Zaas et al., 2010).

1.10 Objectives

This study aims to further characterize the role of the *B. cenocepacia*'s TAAs in the binding to host cells, as the gateways used by these bacteria are still not known. For this purpose lipid rafts are regarded as a viable option for *B. cenocepacia* since they are used by *P. aeruginosa* and other pathogens to adhere, gain entry into cells and, therefore, cause infection. With this in mind adhesion and invasion assays were planned in order to evaluate the BCAM0224 TAA role in the adhesion and invasion of host cells by *B. cenocepacia*. The assays were executed using three different *in vitro* model epithelial cells, two bronchial epithelial cells lines, 16HBE14o$^-$ and CFBE41o$^-$ lines, and the A549 carcinomic human alveolar basal epithelial cell line. In these assays cell monolayers were infected with *B.cenocepacia* K56-2 and *B.cenocepacia* BCAM0224::Tp mutant after being subjected to a methyl-β-cyclodextrin (MβCD) treatment.

In addition, this study also aimed to understand if the BCAM0220 and BCAM0219 operon exhibited the same sort of interaction demonstrated by the SadA and SadB *Salmonella* proteins.

For this purpose, an experiment was planned to understand the workings of the referred operon. In this experiment a BCAM0220 negative mutant and an anti-BCAM0219 antibody were designed in an attempt to shed knowledge on this hypothesis. The mutant and the antibody would be used in adhesion and invasion assays so that it would be possible to understand if the BCAM0220 lipoprotein presence influenced BCAM0219 biogenesis. This experiment would allow us to understand if the BCAM0220 co-expression with BCAM0219 has direct influence on this protein biogenesis, therefore, implying that in this case TAA autotransport is assisted by other periplasmatic proteins.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Two different bacterial strains were used, the wild type *B. cenocepacia* K56-2 and *B. cenocepacia* BCAM0224::Tp mutant, which as a thrimethoprim(Tp) resistance cassette inserted in the BCAM0224 TAA enconding gene.

Both strains were cultured in similar conditions at 37°C with 250 rpm orbital agitation. The *B. cenocepacia* K56-2 strain was routinely cultured in Luria-Bertani Broth (LB) while the mutant strain was cultured in trimethoprim (150 mg/mL) LB supplemented media.

When required for functional studies both strains were grown in 24 wells plates, starting in, an initial OD_{640} of 0.2, in LB media containing NaCl (300 mM) and H_2O_2 (10 mM, for 17 hours under microaerophilic conditions (< 5 % CO_2) at 37°C with 60 rpm orbital agitation.

2.2 Cell lines and cell culture

Three different cell lines were used. 16HBE14o$^-$ and CFBE41o$^-$ cells which are human bronchial epithelial cells, the former are normal lung cells expressing a functional CF transmembranar conductance regulator, the latter are homozygous for the ΔF508 mutation correspondent to a CF airway; and A549 cells which are carcinomic human alveolar basal epithelial cells.

The bronchial epithelial cell lines were ordinarily maintained in Minimum Essential Medium with Earle's salt (MEM) supplemented with 10% fetal bovine serum (FBS), L- Glutamine (0.292 g/L) and penicillin/streptomycin (100 U/mL) in fibronectin/collagen coated flasks in a humidified atmosphere with 5% CO_2 at 37°C. A549 cells were cultured in non-coated flasks in F-12 media supplemented with inactivated 10% FBS and 1% penicillin/streptomycin and maintained under the same atmosphere conditions. When the cells in the flask were 80% to 100% confluent, they were sub-cultured. To sub-culture 16HBE14o$^-$and CFBE 41o- cells the medium was removed , the cells were washed with 3 mL hepes buffered saline (HBS) then, 2 mL PET solution was added(HBS, 10% polyvinylpyrrolidone, 0.2% EGTA and 0.25% trypsin with 0.02% EDTA) and the flask placed in the 37°C incubator to allow the trypsin to work. After 5 minutes the flask was checked for cell detachment aiming to remove > 95% of the cells from the flask surface. Finally, 5 mL medium was added to stop the trypsinization. The cells were repeatedly pipeted to break clumps, centrifuged at 1200 rpm to pellet the cells and resuspended with 4 mL supplemented MEM medium before being

divided into 2 different flasks. A549 cells, after removing the medium, were sub – cultured by, firstly being washed with 3 mL phosphate buffered saline (PBS) and scrapped of the flask surface. Secondly, 4 mL of F-12 media was added to resuspend the cells and divide them into new flasks.

Then, the cell flasks were placed in the incubator at 37°C with humidified atmosphere 5% CO_2.

2.3 Adhesion and invasion assays of human epithelial cells

Both adhesion and invasion assays were carried out on three cell lines: 16HBE14o$^-$, CFBE41o$^-$ and A549 .

Firstly, cells were seeded on 24 wells plates (5 x 10^5 cells per well) in supplemented media for 24 hours at 37°C in humidified 5 % CO_2 containing atmosphere. Then, cells were washed with HBS or PBS, 16HBE14o$^-$, CFBE41o$^-$ or A549 cells, respectively, and maintained in non-supplemented media; before being treated or not with MβCD (500mg/mL) for 30 minutes.

For these assays, *B. cenocepacia* K56-2 and BCAM0224::Tp were used to infect the cell monolayers at a multiplicity of infection (MOI) of 50:1 (bacteria per epithelial cell). After infection the 24 well plates were centrifuged at 700g for 5 minutes. Subsequently, in order to allow bacterial adhesion, the infected monolayers were incubated at 37°C in 5% CO_2 atmosphere for 30 minutes. Afterwards, the cells were washed 3 times with PBS and lysed with 200 µL of lysis buffer (10 Mm EDTA and 0.25 % Triton X-100) for 30 minutes at room temperature.

For the invasion assays, the infected cells were incubated at 37°C in 5% CO_2 for 2 hours to allow bacterial entry. After this, the cells were washed 3 times with PBS and a combination of amikacin and ceftazidime (3 mg/mL each) was added, thereon another 2 hours incubation period followed. To confirm the efficiency of the antibiotic treatment the supernatants were plated. Finally, the cell monolayers were again washed with PBS 3 times and lysed with lysis buffer (10 mM EDTA, 0,25% Triton X-100) for 30 minutes as in the adhesion assays.

The bacteria quantification, in both experiments, was done by serial dilution plating of the cell lysates and the results were expressed as percentage of the bacterial dose applied.

2.4 Plasmids and primers

All plasmids used and generated for this study are shown in **Table 4** as well as a list of primers is shown in **Table 5**. To design these primers *B. cenocepacia* K56-2 genomic sequence was used as a template

Tab. 4: Plasmids generated during the course of this study

Plasmid	Description	Source
pDrive	Cloning vector , Amp^R,Km^R	*Quiagen*
pUC-TP	Derivative of pUC-GM, with ampicilin resistance gene	(Sokol et al .1999)
pK18	Cloning vector, Km^R	
pET23a(+)	Cloning vector, Amp^R	*Novagen*
pET22b(+)	Cloning vector, Amp^R	*Novagen*
pDF1	Cloning vector pDrive with complete *BCAM0220* and flanking intergenic regions	This study
pDF2	Cloning vector pDrive with complete *BCAM0220* and Tp cassette	This study
pDF3	Cloning vector pK18 with fragment $BCAM0219_{223-503aa}$	This study
pDF4	Cloning vector pET23a(+) with fragment $BCAM0219_{620-902aa}$	This study
pDF5	Cloning vector pET23a(+) with fragment $BCAM0219_{66-528aa}$	This study
pDF6	Cloning vector pET23a(+) with fragment $BCAM0224_{633-854aa}$	This study
pDF7	Cloning vector pET-22b(+) with fragment $BCAM0219_{223-503aa}$	This study
pDF8	Cloning vector pET-22b(+) with fragment $BCAM0219_{620-902aa}$	This study
pDF9	Cloning vector pET-22b(+) with fragment $BCAM0224_{633-854aa}$	This study
pDF10	Cloning vector pET22b(+) with fragment $BCAM0224224_{340-638aa}$	This study

Tab. 5: PCR amplification primers.

Primer	5'-3' Sequence
M220KpnF	CGG GGT ACC CAT CCG ACT GAT TCC GAA TCC A
M220HindR	TTT AAG CTT AGA TTT GCG CGG CAA TCG
BCAM0219$_{223-503}$Ndefwd	TTT CAT ATG GCA GTG ACG TAC GAC AAG AA
BCAM0219$_{223-503}$Xhorev	AAA CTC GAG CGC GTT GAC TTG
BCAM0219$_{620-902}$Bam_fwd	CGG GAT CCG CAG TGA CGT ACG ACA AGA A
BCAM0219$_{620-902}$Hind_reverse	CCC AAG CTT CGC GAC GTT CTT GAC TTG C
BCAM0219$_{66-528}$Ndefwd	TTT CAT ATG AAT GGT TCG CTG ACG CTT TGC
BCAM0219$_{66-528}$Hindrev	TTT AAG CTT CAC GTT AAT CTG CCG CTG CCG CTG AA
BCAM0224$_{54-323}$Ndefwd	TTT CAT ATG GCG GAA GTG TGT ACG GCA GAA
BCAM0224$_{54-323}$Xhorev	AAA CTC GAG GGT CTG CAG GTC GAT GT
BCAM0224$_{633-854}$Nde	TTT CAT ATG GTG ACG GTG TCG CAG CTT C
BCAM0224$_{633-854}$Hind	TTT AAG CTT GAC GTT CGT GAT CTG GCG TT
BCAM0224$_{340-638}$Nde	TTT CAT ATG GCA AAC CTG ACC GTC GGC AA
BCAM0224$_{340-638}$Xho	AAA CTC GAG AAG CTG CGA CAC CGT CAC GA

2.5 DNA manipulation techniques

Plasmid DNA was extracted following a Miniprep extraction protocol or using the ZR *Plasmid Miniprep-Classic kit (Zymo research)* by following the manufacturer instructions. Total DNA extraction was done using the *DNeasy Blood & Tissue Kit (Quiagen)* following the manufacturer instructions.

The amplification reactions occurred in a volume of 25 µL each containing 20 ng of *B. cenocepacia* K56-2 DNA, 1x amplification buffer, 1.5 mM $MgSO_4$, 10 mM of primer forward and primer reverse, 2.75 U *Platinum Taq DNA Polymeras*e *(Invitrogen),* 1x or 2x Enhancer solution and *(Invitrogen)* 0,2 mM deoxyribonucleotides. All melting temperatures and extension time were optimized for each set of primers.

2.6 BCAM0220 insertion mutant construction

By PCR a 1.07Kb fragment of the *BCAM0220* gene was amplified from *B. cenocepacia* K56-2 DNA, using primers M220KpnF and M220HindR, which contain the *KpnI* and *HindIII* restriction sites, respectively. The amplification was carried out, after an initial denaturation at 95°C for 2 min, for

35 cycles consisting of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 1min, final extension was carried out at 72°C for 7 min. Then, the PCR product was digested with both *KpnI* and *HindIII* before being cloned into the pDrive cloning vector which was beforehand digested using the same enzymes, as well. From pUC-Tp, the 1.01Kb trimethoprim cassette containing fragment was extracted through *PstI* digestion and, then, inserted in the only *PstI* restriction site of the BCAM0220 fragment.

After, obtaining the construction comprising the pDrive vector, BCAM0220 fragment and the Tp cassette was obtained it was introduced in *B. cenocepacia* K56-2 by eletroporation (25 µF, 2.5 Kv, 200 Ω). The transformants obtained were selected on LB agar supplemented with trimethoprim (150 mg/mL) for 48 hours at 37°C. To distinguish between single and double crossover mutants trimethoprim-resistant colonies were screened by replica plating for kanamycin (600 mg/mL) sensitivity as the pDrive cloning vector has a kanamycin resistance gene. Colonies which only showed trimethoprim resistance were chosen as candidate insertion mutants and their total DNA was extracted.

Lastly, the candidate insertion mutants were characterized by PCR using the above mentioned primers for the fragment amplification, after total DNA extraction. For this confirmation amplification the extension time was changed to up 2 min. This PCR approach would allow the identification of BCAM0220 deficient *B. cenocepacia*.

2.7 Recombinant proteins expression

Different truncated fragments were generated with their specific sizes and primers for generating the polypeptides. The different truncated fragments were created form several TAAs such as BCAM0219 and BCAM0224 using head and stalk frames as a template. All $BCAM0219_{223-503}$, $BCAM0219_{620-902}$ and $BCAM0219_{66-528}$ fragments were generated using the head frame of the BCAM0219 TAA as template and the fragments $BCAM0224_{54-323}$, $BCAM0224_{633-854}$ and $BCAM0224_{340-638}$ the BCAM0224 TAA head and stalk frame, respectively **(Fig.9)**.

The fragments were amplified by PCR using specific primers to introduce *NdeI* and *XhoI* restriction sites to $BCAM0219_{223-503}$, $BCAM0224_{54-323}$, $BCAM0224_{340-638}$ fragments, *NdeI* and *HindIII* restriction sites to $BCAM0219_{66-528}$ and $BCAM0224_{633-854}$ fragments. $BCAM0219_{66-528}$, $BCAM0224_{54-323}$, $BCAM0224_{633-854}$ and $BCAM0224_{340-638}$ fragment amplification occurred following a scheme of denaturation at 95°C for 30s, annealing at 57°C for 30s and extension at 72°C for 1 min repeated for 35 cycles and final extension at 72°C for 7 min; after an initial denaturation at 95°C for 2 min.

The amplification of fragments BCAM0219$_{66-528}$ was carried out in similar conditions except for the annealing which occurred at 53°C for 30s.

Fragment BCAM0219$_{620-902}$ was amplified by PCR using specific primers to introduce *BamHI* and *HindIII* restriction site. Firstly, the BCAM0219$_{620-902}$ PCR product was cloned into pK18 cloning vector. The resulting plasmid was first transformed by eletroporation (25 µF, 2.5 Kv, 400 Ω) into to the non-expressing *E. coli* αDH5 which was incubated in the presence of LB ampicilin (150 mg/mL) containing media plates at 37°C overnight. Before, plating a mixture of isopropyl-1-thio-β-D-galactoside (IPTG) (25 mg/mL) and X-GAL (25 mg/mL) was added to the media plates to allow transformant colonies selection by α- complementation as pK18 has a *lacZ* gene. Then, all PCR products were cloned into pET22b(+) or pET23(+) cloning vectors.

The resulting plasmids were transformed by eletroporation (25µF, 2.5 Kv, 400Ω) into *E. coli* αDH5 which was incubated in the presence of LB ampilicin (150mg/L) containing media plates at 37°C overnight.

Thereafter, the plasmidique DNA was extracted and the plasmids coding for the fragments were checked for the specific fragment presence by restriction enzyme digestion before being transformed into *E. coli* BL21(DE3) that, also, was incubated in LB plates supplemented with ampicilin (150 mg/mL) at 37°C overnight. Then, the strain was grown overnight with 250 rpm orbital agitation at 37°C in LB media containing ampicilin (150mg/mL), for subsequent overexpression.

For overexpression, the strain was grown to an initial OD_{640nm}= 0.1 previous to inoculation into fresh supplemented media. After this, the strain was grown, for approximately 2 hours, to an OD_{640nm}= 0.6-0.8 followed by 1mM, 0.2mM and 0.5mM IPTG induction. Later samples were taken, after 2 hours, 3 hours and 4hours growth at an OD_{640nm}=0.5. The samples taken were maintained in sample buffer at -20°C.

Afterwards, recombinant protein overexpression was verified by whole-cell protein electrophoresis.

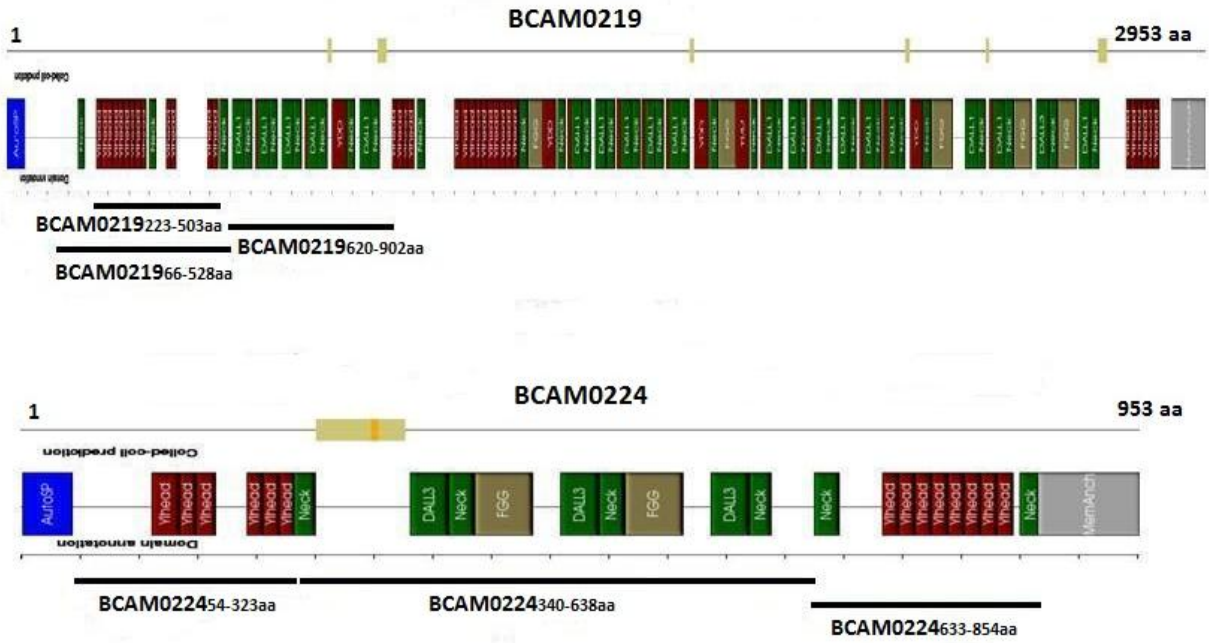


Fig. 9: Generated fragments TAA region overview. Blue represents the peptide signal. Gray represents the membrane anchor. Red represents the YadA head like protein domains. Green represents the neck like domains. (http://toolkit.tuebingen.mpg.de/dataa, 30/102014, 17h).

2.8 Whole-cell protein electrophoresis

In order to verify recombinant protein overexpression, SDS-Polyacrylamide gels (SDS-PAGE) were cast with the appropriate percentages of polyacrylamide, according to the expected recombinant protein molecular weight. 5% stacking gels and 15% resolving were prepared by mixing the respective amount of 30% acrylamide *(Sigma-Aldrich)*, 10% ammonium persulfate, 10% SDS, TEMED, water and 1.5 or 1M Tris for the resolving and stacking gels, respectively.

The electrophoretic conditions used were 150V for about 1h30min. Then, the SDS-PAGE was stained with Commassie Blue.

2.9 Statistical analysis

All statistical data analysis was done using *Microsoft Excel* (*Microsoft*) by computing the percentage of bacterial adhesion and invasion for both MβCD treated and untreated cells. The results were expressed as a ratio of the wild type and corrected with bacterial dose applied. The ANOVA test for significance was used for relative comparisons, considering a p-value<0.05 as statistical significant.

3. Results

3.1 *B.cenocepacia* adherence and invasion assays of epithelial cells.

To access whether lipid rafts are involved or not in adherence and invasion of airway epithelial cells by *B. cenocepacia* K56-2*,* adhesion and invasion assays were performed using three different *in vitro* model bronchial epithelial cells, 16HBE14o- and CFBE41o- , which have a CF and non-CF phenotype, respectively, and, A549 carcinomic human alveolar basal epithelial cells. Cell monolayers were infected with *B. cenocepacia* K56-2 and *B. cenocepacia* BCAM0224::Tp mutant after being subjected to a lipid depletion treatment with MβCD (500 mg/mL). All adherence and invasion assays for all cell lines were done with both treated and untreated cells in order to allow comparison between the different conditions.

3.1.1 CFBE41o- adhesion and invasion assays

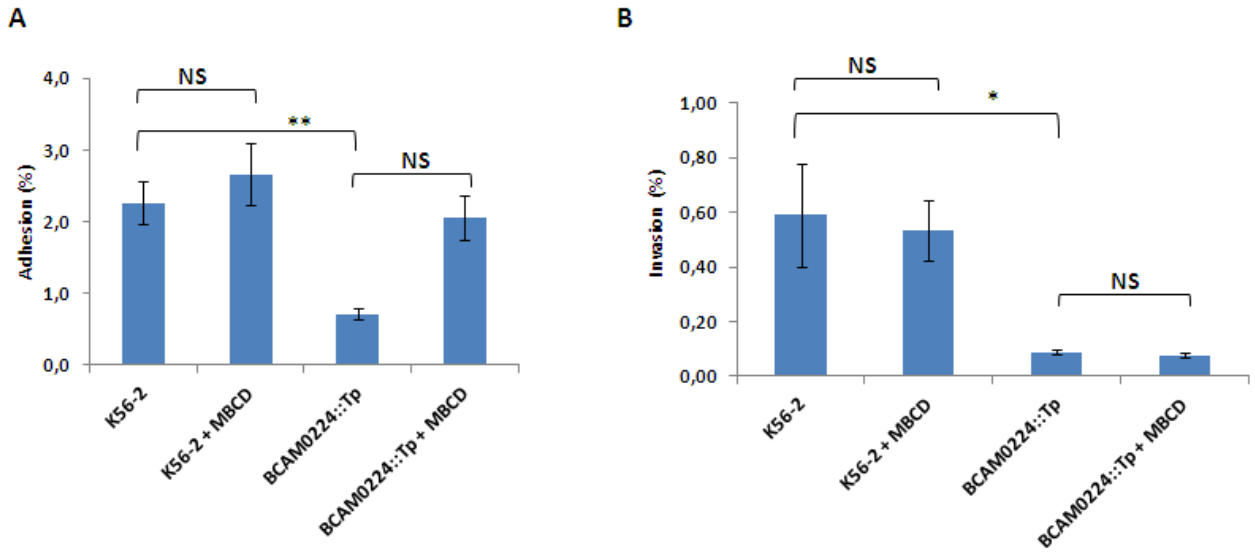


Fig. 10: Effect of MβCD (10 mM) CFBE41o- host cell treatment on *B. cenocepacia* K56-2 (K56-2) and BCAM0224::Tp mutant capacity of adhesion **(A)** and invasion **(B)**. **A)** In both *B. cenocepacia* K56-2 (K56-2+MβCD) and mutant (BCAM0224::Tp+MβCD) infection of treated CFBE41o$^-$ cells the percentage of adhesion showed no statistical difference (NS). Only the mutant's (BCAM0224) adhesion to untreated cells was significantly decreased (**p<0.01). **B)** Both *B.cenocepacia* K56-2 (K56-2+MβCD) and mutant (BCAM0224::Tp+MβCD) invasion of treated CFBE41o$^-$ cells shows no statistical difference (NS). Only the mutant's (BCAM0224) invasion of CFBE41o$^-$ untreated cells was significantly decreased ($^*p$<0.05). Results are shown as percentage of the initial dose applied.

Both adhesion and invasion percentages for this cell line assays are depicted in **Fig.10**. As shown **Fig.10A**, the BCAM0224-negative mutant adhered less efficiently than the wild type strain

to the untreated cells (**p<0.01). Nevertheless, in contrast, to these results, when in the presence of treated cells both strains seem to demonstrate increased adherence to host cells, but there was no statistical difference in the results (NS). Furthermore, the invasion assays, in **Fig.10B**, the results indicate that both *B. cenocepacia* K56-2 and *B. cenocepacia* BCAM0224::Tp mutant demonstrate equal percentages of invasion (NS) when comparing both strains invasive ability whether the cells were treated or not. Additionally, the BCAM0224-negative mutant shows a decreased percentage of invasion (*p<0.05).

3.1.2 A549 adhesion and invasion assays

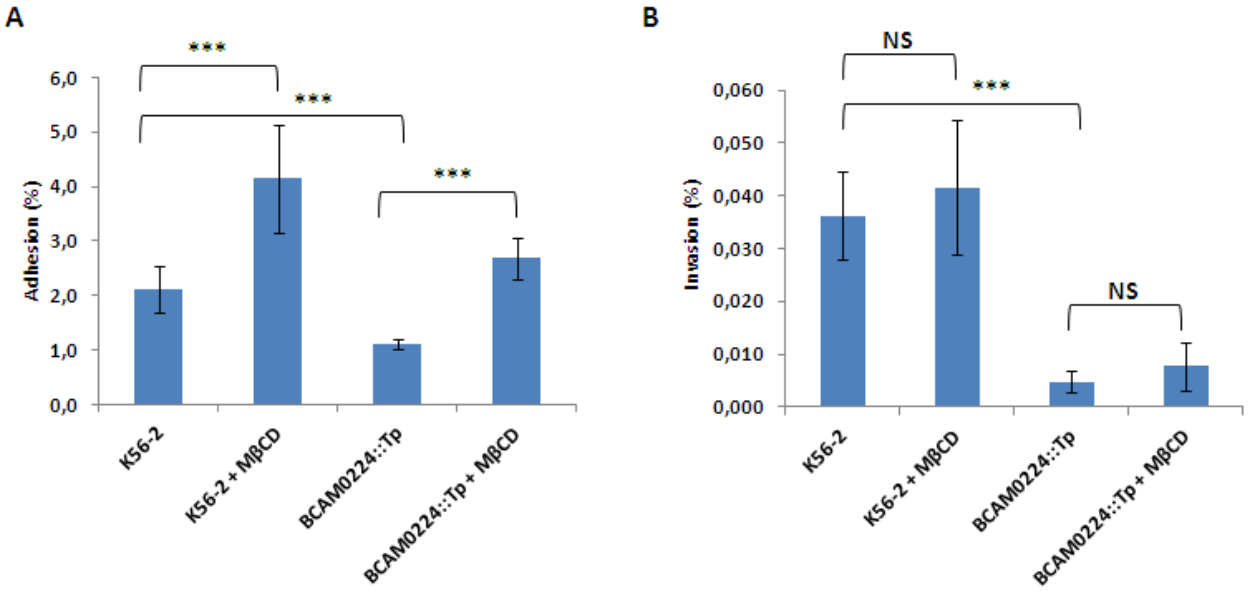


Fig. 11: Effect of MβCD (10 mM) A549 host cell treatment on *B. cenocepacia* K56-2 (K56-2) and BCAM0224::Tp mutant capacity of adhesion **(A)** and invasion **(B)**. **A)** In both *B. cenocepacia* K56-2 (K56-2+MβCD) and mutant (BCAM0224::Tp+MβCD) infection of treated A549 cells the percentage of adhesion was significantly increased (***p<0.001). Only the mutant's (BCAM0224) adhesion to untreated cells was significantly decreased. **B)** Both *B.cenocepacia* K56-2 (K56-2+MβCD) and mutant (BCAM0224::Tp+MβCD) invasion of treated A549 cells shows no statistical difference (NS). Only the mutant's (BCAM0224) invasion of A549 untreated cells was significantly decreased (***p<0.001). Results are shown as percentage of the initial dose applied.

In **Fig.11** are presented the results of the assays performed with the A549 cell line. The results in **Fig.11A** show a marked increase of adhesion percentage for *B. cenocepacia* K56-2 and *B. cenocepacia* BCAM0224::Tp mutant in both MβCD treated cells (***p<0.001). When A549 cells lose lipid rafts the capacity of *B. cenocepacia* wild type and mutant, became enhanced. However, in invasion **(Fig. 11B)** the results show no significant differences in the *B. cenocepacia* K56-2 and *B. cenocepacia* BCAM0224::Tp mutant capacity of invasion when A549 cells lose lipid rafts. In

addition, as shown in **Fig. 11A** and **11B** the BCAM0224-negative mutant adhered and invaded untreated cells less efficiently (***p<0.001).

3.1.3 16HBE14o- adherence and invasion assays

The adhesion and invasion assays for the 16HBE14o$^-$ cells yielded variable results. Despite several experiments a valuable result couldn't be achieved. Therefore, it was not possible to understand if the MβCD cells treatment affected the bacteria's adhesion and invasion ability.

3.2 BCAM0220 insertion mutant construction

The *BCAM0220* gene, which encodes a lipoprotein in the putative adhesion cluster, was chosen for the construction of this insertion mutant because this gene was demonstrated to be in the same operon as the *BCAM0219* gene, suggesting a possible interaction between the two; as seen for the *S. enterica* SadB lipoprotein (Grin et al., 2014).

The methodology for the mutant construction was described in the Materials and Methods chapter, the complete *BCAM0220* gene with flanking intergenic regions was amplified by PCR, which was cloned into the pDrive cloning vector, and, then the trimethoprim cassette containing fragment was inserted in the only *PstI* restriction site of the BCAM0220 fragment. This construction was then introduced in *B. cenocepacia* K56-2 by eletroporation. Finally the identification of BCAM0220 deficient *B. cenocepacia* was executed by PCR. As the plasmid pDrive is non replicative in *B. cenocepacia*, if the double crossover recombination occurs, we'll obtain the amplification of the BCAM0220 fragment (1.07 Kb) plus the trimethoprim cassette (1.01 Kb), performing an amplification of 2.08 Kb. However, if only a single recombination event occurs pDF2 would be integrally inserted into the *B. cenocepacia* genome and the *BCAM0220* gene remains intact, leading on two amplification products, one with 1.07 Kb , and another with 2.08 Kb.

Unfortunately, the BCAM0220 insertion mutant with double recombination was not obtained after many attempts, only the first recombinant step has occurred **(Fig.12)**. As presented in **Fig.12**, it is possible to note the presence of two stained bands, a less stained band marked at the 2 Kb size and a strongly stained band marked at approximately 1 Kb. Nevertheless, the excepted result should be a single band at the 2 Kb , confirming the insertion of the trimethoprim cassette in the *BCAM0220* gene.

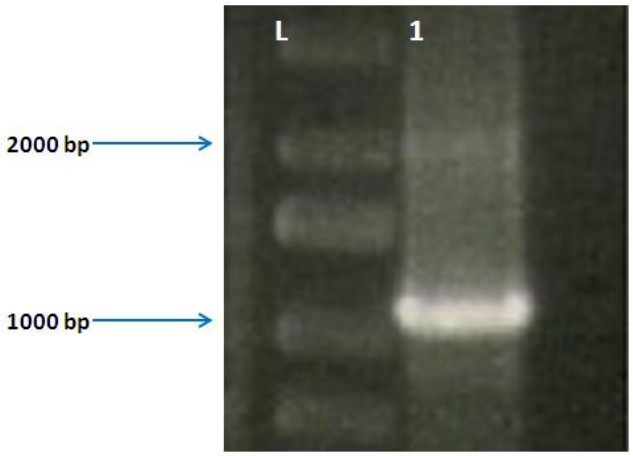


Fig. 12: Screening of a BCAM0220 insertion mutant. Lane 1- Colony 1 with 1000bp and 2000bp. L- 1Kb DNA ladder.

3.3 BCAM0219 and BCAM0224 truncated recombinant proteins construction

Several head and stalk frames from the BCAM0219 and BCAM0224 TAAs were used as templates in order to understand which was better to facilitate the construction of the truncated proteins. Then, followed the overexpression of the expected truncated protein, after the different DNA fragments were obtained, subsequently cloned into the pET22b(+) or pET23(+) cloning vectors and introduced into *E. coli* BL21(DE3).

Unfortunately, overexpression of the truncated proteins was not achieved and the reasons possible for such will be discussed in the following chapter. Additionally, the results for the proteins for which the overexpression step was reached are presented in **Fig.13** and **Fig.14** that shows the cast SDS-polyacrylamide gels for the truncated proteins $BCAM0219_{223\text{-}503}$, $BCAM0219_{620\text{-}902}$, $BCAM0219_{66\text{-}528}$ and $BCAM0224_{633\text{-}854}$. As is shown in **Fig. 13** and **Fig.14** overexpression was not obtained as no distinctly marked yield of protein is observed in comparison with the initial time sample **(T0)** that was taken before the IPTG induction. The expected proteins $BCAM0219_{223\text{-}503}$ and $BCAM0219_{620\text{-}902}$ would have a predicted size of 29KDa and 30KDa, respectively, and the remained truncated proteins would have approximately the same size.

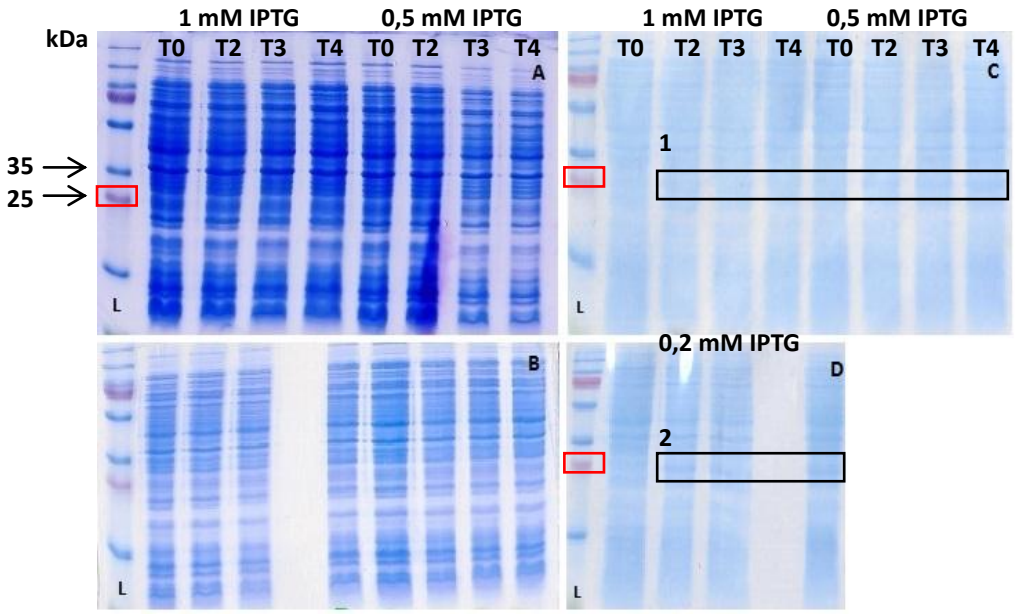


Fig. 13: SDS-PAGE BCAM0219$_{223-503}$ and BCAM0219$_{620-902}$ protein analysis. **A** to **B** - BCAM0219$_{223-503}$ protein SDS-PAGE overexpression analysis. **C** to **D** - BCAM0219$_{620-902}$ protein SDS-PAGE overexpression analysis. 1 to 2- BCAM0219$_{620-902}$ protein low overexpression as a result of the 1, 0,2 and 0,5mM IPTG induction. T0- initial time sample before IPTG induction; T2- 2 hours after IPTG induction sample; T3- 3 hours after IPTG induction sample; T4- 4 hours after IPTG induction sample; L- ladder.

Also, as stated before, in **Fig.13 A** and **Fig.14 B** there is no BCAM0219$_{223-503}$ protein overexpression as all whole cell protein profiles (T2,T3,T4), in different IPTG conditions used, are identical to the initial time (T0) sample in which no protein overexpression was induced. However, in **Fig. 13 C** and **D** low protein overexpression can be observed; nevertheless the protein overexpressed does not exhibit the predicted size of 30KDa but instead a size of 25KDa, therefore, indicating that this is not the protein expected.

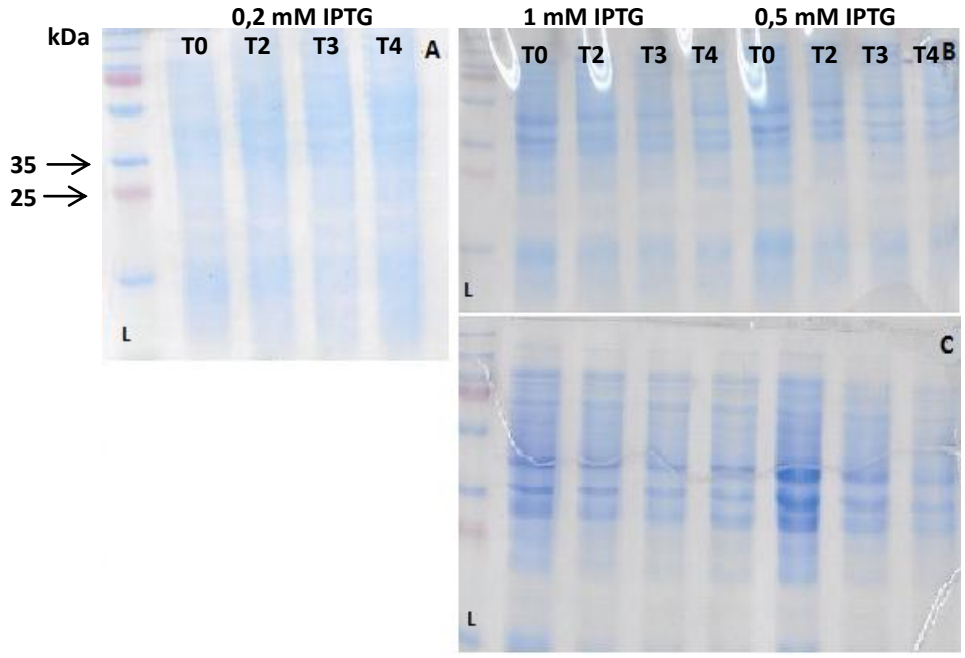


Fig. 14: SDS-PAGE BCAM0219$_{66-528}$ and BCAM0224$_{633-854}$ protein analysis. **A** - BCAM0219$_{66-528}$ protein SDS-

PAGE overexpression analysis; **B** to **C** – BCAM0224$_{633\text{-}854aa}$ protein SDS-PAGE overexpression analysis. T0- initial time sample before IPTG induction; T2- 2 hours after IPTG induction sample; T3- 3 hours after IPTG induction sample; T4- 4 hours after IPTG induction sample;L- ladder.

Fig.14 shows that there was no overexpression for the expected BCAM0219$_{66\text{-}528}$ **(Fig.14A)** and BCAM0224$_{633\text{-}854}$ **(Fig14B)** truncated proteins, as well, because, as before, the whole-cell protein profiles are identical to the T0 sample taken for all the IPTG conditions utilized.

4. Discussion

4.1 Lipid rafts do not interfere in the adherence and invasion of epithelial cells by *B. cenocepacia*.

As mentioned before lipid rafts act as a point of adherence and invasion to host cells for various pathogens, such as *P. aeruginosa,* the most common pathogen in CF patients, therefore, lipid rafts would be a probable gateway to *B. cenocepacia* invasion of host cells through the TAAs binding.

However, the results obtained through the *B.cenocepacia* K56-2 and BCAM0224::Tp mutant adhesion and invasion assays revealed that lipid rafts do not interfere in the ability of these bacteria to either adhere and or gain entry into host cells despite their phenotype. Furthermore, surprisingly, both wild type and mutant strain show, in some cases, an increased ability of adhesion in the presence of CFBE41o- and A549 treated cells whereas the percentages of invasion for both cell lines show no significant differences.

Moreover, in the presence of disrupted lipid rafts the binding of either *B. cenocepacia* K56-2 or BCAM0224::Tp mutant should have been impaired if lipid rafts were to be used as binding sites. In contrast, the absence of the BCAM0224 TAA and the disruption of the lipid rafts did not disturb the bacteria's capacity of adhesion and invasion. Even though, the wild type strain percentage of adhesion and invasion is higher than that of the mutant strain, as expected. The compared percentages of invasion and adhesion for both *B. cenocepacia* K56-2 and the BCAM0224::Tp mutant for treated and non-treated cell indicate the bacteria's ability to keep invading host cells in the absence of lipid rafts.

Overall, these bacteria TAAs were able to mediate the binding and invasion of host cells in the absence of lipid rafts, therefore, demonstrating, that, in contrast with many other organisms, lipid rafts are not used as gateways by *B. cenocepacia* adhesins.

4.2 BCAM0220 negative mutant construction

As seen in the results chapter the intended second recombination event did not happen and, therefore, it was not possible to obtain the BCAM0220::Tp mutant. Homologous recombination is a process that results in genetic exchange between homologous DNA sequences from two different sources. The pDF2 generated plasmid which was introduced by eletroporation into *B. cenocepacia* K56-2 and the chromosomes of *B. cenocepacia* are the two different sources.

Nevertheless, before discussing the mechanisms of recombination of the introduced plasmid this same transferred DNA faces three different fates: degradation by restriction enzymes, replication by itself or it may recombine with the host chromossome. For this study the objective was to achieve the last option, the recombination of the Tp cassette interrupted *BCAM0220* gene into one of the chromosomes of *B. cenocepacia*. This method is based on the integration of the generated plasmid pDF2 into a chromosome by a single crossover recombination event that is followed by resolution of the integrated DNA by a second recombination event which results in the replacement of the original *BCAM0220* gene with the inactivated gene, i.e., the Tp cassette interrupted *BCAM0220* gene. However, in this study, the PCR analysis of a mutant candidate revealed that only a single crossover happened as a 1071bp fragment was amplified indicating the presence of an original wild type gene. Even though a 2000bp fragment was, as well, amplified, in order to obtain mutant the only amplified sequence should be that of the disrupted *BCAM0220* gene signalling the occurrence of a second crossover and, therefore, the replacement of the original *BCAM0220* gene. Although, the construction of the BCAM0220 insertion mutant was not successful in this study; this method has already provided stable mutants as for example the BCAM0223 negative mutant (Mil-Homens et al., 2012), which indicates this method as appropriate to the construction of this mutant.

Furthermore, a trimeric lipoprotein was already described to aid in the biogenesis of an autotransporter in *Salmonella* (Grin et al.2014), therefore, if the mutant had been constructed it would help to learn if the BCAM0220 outer-membrane lipoprotein assists the TAA BCAM0219 in its binding or biogenesis as both were demonstrated to be an operon in the adhesin putative cluster.

4.3 BCAM0219 and BCAM0224 truncated recombinant protein construction

In this study, the BCAM0219 and BCAM0224 recombinant truncated protein construction was unsuccessful, as only low or no overexpression of the target truncated proteins was achieved. There are several reasons that impede the expression truncated proteins and the problems often lie in a harmful effect that the heterologous protein causes on the cell. When low or no recombinant protein production happens the problem, usually, lies with protein toxicity, which could interfere with the proliferation of the microorganism resulting in a slow growth rate, or codon bias, that leads to the depletion of low abundance t-RNAs resulting in amino acid misincorporation and/or truncation of the polypeptide which, in turn affects the heterologous protein expression, and lastly, the recombinant protein might have been degraded by the cell

mechanisms as it may have been recognized as a faulty protein or lacking a proper signal specifically directing it (Rosano & Ceccarelli, 2014).

Several strategies can be employed to overcome the lack of recombinant protein expression, in order to increase the protein yield as, for example, changing the vector, host and the culture of the recombinant host strain, co-expression of other genes (e.g. another protein that stabilizes the desired protein) and changing the gene sequences, which may help increase expression and the proper folding of the desired protein (Rosano & Ceccarelli, 2014). Therefore, in this study as overexpression of all protein of interest was not yielding results, the initially used vector pET23(+) was changed for the pET22(+) cloning vector which encodes a signal sequence specifically directing the protein to the membrane and distinct frame sequences were used as templates which resulted in different generated fragments listed in the Materials and Methods chapter.

Although, after using the pET22(+) vector, low protein overexpression could be observed in Fig.13 C and D in boxes 1 and 2 of the results chapter, the protein overexpressed is not the expected BCAM0219$_{620\text{-}902}$, but, instead another protein. Such occurrence can be explained by the out of frame construction of the pDF8 generated plasmid which resulted in the translation and expression of a foreign protein as the available ORF was not enconding the protein of interest, but, instead a different protein.

Lastly, successfully obtaining the BCAM0219 antibody would enable the study of the possible interaction between the BCAM0220 protein and the BCAM0219 TAA. As it would allow us to understand how the absence of the BCAM0220 protein would affect the BCAM0219 TAA biogenesis, and, therefore, the antibody of interest would reveal the presence or absence of the BCAM0219 protein at *B. cenocepacia*'s membrane.

5. Conclusion and future perspectives

In conclusion, this study allowed to understand that the lipid rafts do not interfere in the *B. cenocepacia* adhesion and invasion of CF airway epithelial cells, in contrast, with microorganisms as *P. aeruginosa,* the most prevalent pathogen in CF patients, *B. abortus* and *N. meningitidis* (Jimenez de Bagues et al., 2005; Mikaty et al,2009; Vieira et al., 2010;Watarai et al., 2002). So, in future studies the aim might be to search for a novel receptor candidate that helps to further understand the binding of the TAAs and the way this adhesins interfere in *B. cenocepacia* infection. A viable candidate to study would be the tumour necrosis factor receptor 1 (TNRF1) as B*. cenocepacia* BC-7, a ET-12 lineage strain binds to this receptor through an undefined bacterial ligand that is not cable pili (Sajjan et al., 2008).

Furthermore, in what concerns the BCAM0219 antibody construction a new plasmid should be generated with pET22(+) vector in addition to the $BCAM0219_{620\text{-}902}$ introduced in the correct frame.

Finally, the objective of this study to understand if cooperation between the both BCAM0220 and BCAM0219 proteins exist was not fulfilled as; unfortunately both the needed antibody and mutant were not successfully generated.

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